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Gene Expression Profiling During Grape Leaf Development and Senescence by High Density Filters

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Abstract

Leaf photosynthesis in grapevines peaks approximately 30 to 40 days after unfolding and declines thereafter. In order to achieve high quality fruit is important that during the ripening stage the vine's photosynthetic capacity is not diminished due to the leaf senescence. To study grapevine leaf senescence at the molecular level, a transcription profiling analysis during leaf development and senescence was undertaken. A cDNA macroarray containing about 2300 putative unigenes was constructed and hybridized with RNA isolated from *Vitis vinifera* 'Pinot noir' leaves 7, 88 and 131 days-old. Data analysis focused on genes involved in the photosynthetic process or belonging to the proteinase family. At this stage of leaf senescence two gene categories were mainly affected: the chlorophyll a/b binding (CAB) genes and the cysteine-proteinases genes. While CAB transcripts decrease in their abundance, cysteine-proteinases are activated.

INTRODUCTION

Leaf development and senescence are biological processes under tight genetic control and characterized by an increase in the photosynthetic rates during leaf expansion and a decline during leaf senescence (Bertamini and Nedunchezhian, 2002). To obtain high quality fruit it is important that during the ripening stage the vine's canopy does not lose photosynthetic capacity. Vineyard management practices such as pruning or fertilization can slow down leaf aging but often this will not entirely solve the problem.

High-throughput sequencing of expressed sequence tags (ESTs) and gene expression analysis, on a genome-wide scale, have opened new possibilities to shed light on complex biological processes such as leaf senescence (Andersson et al., 2004; Bhalerao et al., 2003). Understanding the molecular biology governing the physiology of such a process may help in maintaining the efficiency of the plant for a greater duration of the season.

During the last three years we set up and characterized 6 cDNA libraries from different grape organs and collected a large number of ESTs which are stored in a publicly available database (http://www.ismaa.it/) and deposited also in GeneBank (http://www.ncbi.nlm.nih.gov/dbEST/index.html). A subset of the cDNA clones from the leaf, shoot, inflorescence bud and berry cDNA libraries (ca. 2300 unique sequences) have been spotted on high density nylon filters and probed with RNA isolated from grapevine leaves at different developmental stages. In this paper we present the results of the

analysis of the expression data for those genes that are involved specifically in photosynthesis and in protein degradation, two processes which are strongly affected during leaf senescence.

MATERIALS AND METHODS

The 8th leaf of *Vitis vinifera* L. 'Pinot noir' from 10-year-old vines grafted to '3309C' grown under field conditions with upright growing shoots in San Michele all'Adige (2002) was collected at 3 developmental stages: expanding (7-days-old), mature (88-days-old) and senescent (131-days-old).

The 4010 ESTs clones derived from 'Pinot noir' leaf, bud, and berry and 'Regent' shoot, inflorescence cDNA libraries were PCR amplified and double spotted on high density nylon filters (7.3 cm x 11.5 cm) in a 5 x 5 pattern. The clustering of these ESTs had previously revealed that they correspond to 2320 unique sequences. Total RNA was isolated from the leaf samples according to Moser et al., 2004. For the labelling of each cDNA probe 25 µg of total RNA and [^{33}P]-α-dCTP were used in a reverse transcription reaction. Filters hybridization was carried out at 65°C with a standard procedure (Sambrook et al., 1989). Twelve replicates (including the double spots) of each data point were obtained for each leaf developmental stage.

Hybridization signals were quantified by the programme Arrayvision (Imaging Research Inc.) and corrected for local background. Clones for which 90% of the hybridization signals were below a threshold value (95th percentile of the intensities observed in the empty spots) were discarded from the rest of the analysis. Signal intensities were then normalized by using as spiking control the human nebulin gene, a transcript not present in grapevine and added at a concentration of 0.01% of the poly(A)$^+$RNA amount used for each probe synthesis. Target spots of human nebulin were spotted on the filters at growing concentrations (0.01 ng µL^{-1}, 0.10 ng µL^{-1}, 10 ng µL^{-1} and 100 ng µL^{-1}) and a linear regression was performed on nebulin data (log of median expression of the array versus log of median expression of the overall nebulin data) for each array. Intercept and regression coefficient of each filter were then applied to the whole data set of the same filter. The result of the normalization process was validated by comparison with the transcript concentration of four genes determined by quantitative real time PCR. As gene expression value for each clone was taken the median value of the 12 data replicates.

RESULTS AND DISCUSSION

A high density cDNA array was constructed by double spotting 4010 PCR amplified clones derived from 5 different grape organs, namely, leaf, berry, bud, shoot and inflorescence. About 80% of these clones were previously subjected to single-pass 5' sequencing as part of our EST sequencing project and have been clustered into 2320 contigs (ca. 40% redundancy). We were able to annotate about 75% of these temptative unigenes by sequence comparison against the non-redundant protein NCBI database using the programme PSI-BLAST. Although the amplified cDNA on the macroarray were corresponding only in part to leaf sequences, our hybridization results revealed that the high majority of the clones gave a hybridization signal.

Since we were mainly interested in understanding the molecular biology underlying the severe morphological and physiological changes that occur going from a young leaf to an old one with respect to the mature stage, we decided to primarily focus our analysis on selected categories of genes expressed at the mature stage as a reference.

The genes chosen to be investigated in this study were those related to the photosynthesis and those belonging to the proteinases family since these two functional categories are known to be dramatically affected by the senescence process (Andersson et al., 2004; Gepstein et al., 2003). Although our macroarray does not include all the genes involved in these processes, our data are still highly valuable for two reasons: the paucity of studies on leaf ontogeny and senescence of perennial species and the often strictly correlated expression profiles shown by genes belonging to the same metabolism.

The process of leaf senescence involves a shift in a leaf's function: its contribution to carbohydrate production in the plant diminishes because of a decline in its photosynthetic activity and the remobilisation of nitrogen, phosphorus and metals to younger leaves or to other compartments (Quirino et al., 2000). In our study (Fig. 1) we found two different behaviours in the transcripts level of CAB genes on one hand and the Rubisco small subunit and Photosystem II (PSII) and I (PSI) genes on the other hand. Going from the young to the senescent stages the average expression of the CAB genes shows a large decrease becoming half its initial value whereas the constituents of the PSII, PSI and the Rubisco small subunit show little changes. This result would indicate that the CAB transcripts have a peak concentration in the young leaf followed by a constant decrease thereafter and that the other components of the photosynthetic apparatus considered in this study were not yet affected by the onset of senescence. These data are in good agreement with the physiological data collected on the same samples: the activity of electron transport through the whole chain (H_2O > MV) measured on isolated thylakoid membranes declined only 15% from the mature to the senescent leaves (data not shown). As depicted in Fig. 1 a decrease in gene expression of the CAB genes and the Rubisco small subunit, much more noticeable for the CAB genes, was observed between the young stage and the mature one. A partial (CAB genes) or full recovery (Rubisco) in the transcripts concentrations occurred then going from the mature to the senescent stage. This large decrease in expression could be at least partially explained by a strong climate change (thunderstorm with hail) that occurred a few days prior to sampling the leaves for the mature stage analysis. Apparently this stress highly affected CAB and Rubisco transcripts accumulation but only minimally affected the expression of the other photosynthetic components.

Proteoloytic degradation takes place in all senescing plant organs in order to efficiently degrade cell components and re-allocate the nutrients to other organs. Accordingly an increase in the mRNA levels of specific proteases has been observed during senescence both in the model plant *Arabidopsis* (Gepstein et al., 2003) and in autumn aspen leaves (Bhalerao et al., 2003). In all studied senescing systems cellular proteolysis is carried out by similar mechanisms including aspartic- cysteine-proteinases and the ubiquitin degradation pathway. In our filters were included cDNA clones belonging to the cysteine-, subtilisin-, metallo- and ubiquitin specific proteinases. Three classes of proteinase transcripts out of four appeared to accumulate in the leaves from 88 to 131 days of age, even though not at the same rate (Fig. 2). The cysteine class of proteinases increased 150% with respect to the subtilisin and ubiquitin specific proteinase classes. The gene for a metalloproteinase showed instead a large decrease in its abundance from the mature to the senescent stage. Our results are in agreement with others that have identified specific cysteine-proteinases among the senescence-associated genes (SAGs), genes that are upregulated during senescence (Chandlee, 2001). Moreover, as previously reported for aspen leaves during autumn (Bhalerao et al., 2003) no major activation of the ubiquitin specific proteinase was observed in grape leaves.

In conclusion our ESTs-based macro-array has revealed to be a valuable tool for studying gene expression during grapevine leaf development and particularly in the senescent leaf. Our transcriptional analysis, focused on genes involved in the photosynthetic process or belonging to the family of the proteinases, has demonstrated that at this stage of leaf senescence two gene categories were mainly affected: the CAB genes and the cysteine-proteinases genes. While CAB transcripts decrease in abundance, cysteine-proteinases appear to be activated. It is our intention to extend this type of investigation to older leaves in order to see when a decline in the transcripts level of the other photosynthetic genes occurs.

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Figures

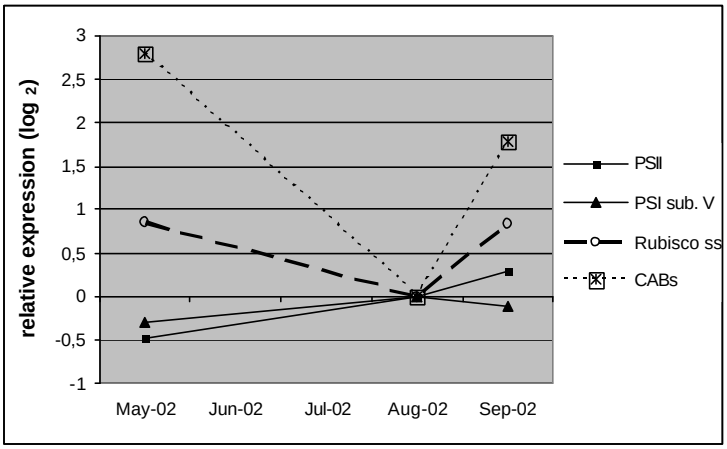

Fig. 1. Gene expression pattern of photosynthesis-related genes in grape leaves of different ages (7, 88 and 131 days from unfolding). PSII: psbT (Acc.: CF607382),10kDa polipeptide (Acc.: CF609227), psbW (Acc.: CF607350), oxygen evolving enhancer protein 1 (Acc.: CF609404). PSI: subunit V precursor (Acc.: CF607564). Rubisco small subunit (Acc.: CF608461). CABs (chlorophyll a/b binding genes): light-harvesting chlorophyll a/b binding protein (Acc.: 609601 and 606496, 606408, 607287 and 606883, 606963), CAB binding protein 40 (Acc.: 606286 and 607092 and 606632). The average levels of expression of the different categories are reported as the logarithm to the base 2 of the ratio between the expression value at that age and the 88-day-old leaves expression value to the reference.

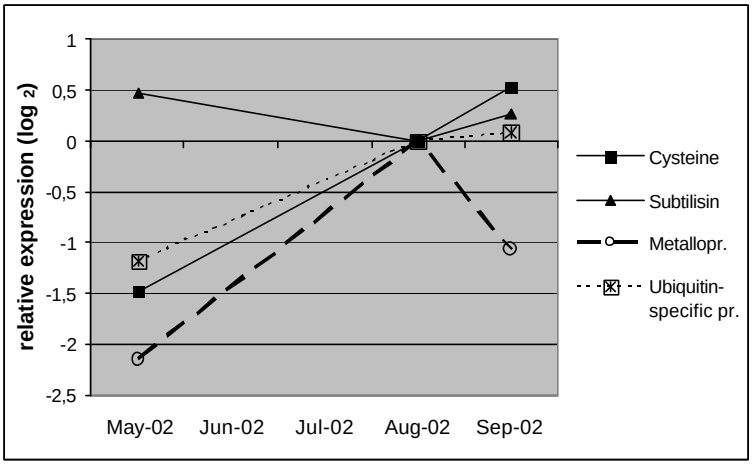

Fig. 2. Gene expression pattern of several classes of proteinases genes in grape leaves of different age (7, 88 and 131 days from unfolding). Cysteine: (Acc.: CF606407, 609367, 609241, 609618 and 609506, 610086). Subtilisin: (Acc.: CF609020 and 609479, 609190 and 608895). Metalloproteinase (Acc.: CF607015). Ubiquitin-specific proteinase (Acc.: CF606767). The average levels of expression of the different categories are reported as the logarithm to the base 2 of the ratio between the expression value at that age and the 88-day-old leaves expression value as the reference.