In Silico ANALYSES OF THE ETHYLENE RESPONSE FACTOR (ERF) AND CELL-WALL ENZYME GENE FAMILIES ASSOCIATED TO RIPENING IN APPLE.

César Luís Girardi[1]*, Vera Quecini[1], César V. Rombaldi[2], Norma Machado da Silva[1], Renata da Silva Moura[2], Tatiane Timm Storch[2], Paula M. Nobile[3], François Laurens[3], Mondher Bouzayen[4]

[1]Embrapa Uva e Vinho - Caixa Postal 130, 95700-000, Bento Gonçalves, RS - Brazil; [2]Universidade Federal de Pelotas, Faculdade de Agronomia Eliseu Maciel, Departamento de Ciência e Tecnologia Agroindustrial –s/n° caixa postal 354, 96010-900, Pelotas, RS - Brazil; [3]UMR 1259 Génétique et Horticulture (GenHort) Angers, France;
[4]UMR 990, Génomique et Biotechnologie des Fruits, INP, ENSAT, INRA, F-31320 Castanet Tolosan, France

Introduction

Flesh fruit ripening is a complex developmental process that involves the formation of taste, aroma, establishment of skin color and fruit softening by cell wall hydrolysis. Physiological, biochemical and molecular evidences indicate that ethylene is the principal hormone controlling this developmental process. It exerts its action on several targets including its own biosynthesis, its perception by the target-cells via ethylene receptors (ETRs), a signal transduction network consisting of positive and negative regulators, and finally on the regulation of target-gene expression by transcription factors, such as ethylene response factors (ERFs) (STEPANOVA & ALONSO, 2009). In higher plant genomes, ERF is present as a superfamily of transcription factor genes defined by the presence of the APETALA2/ERF domain, a conserved DNA-binding sequence (NAKANO *et al.*, 2006). In fruit species, ERFs are associated to flavor biosynthesis and texture modification during ripening (BAPAT *et al.*, 2010). In the present work, we established a genetic framework for ERF and cell-wall proteins associated to the changes in fruit quality, texture and physiological post-harvest disorders in *Malus* using bioinformatic tools and comparative genomic approaches. The preliminary information obtained by *in silico* analyses is currently being validated by *in vivo* gene expression studies.

Material and Methods

Database Searches and Alignments

Homologs of *Arabidopsis thaliana* ethylene response factors (ERF) and cell-wall enzyme genes were identified by BLAST searches (ALTSCHULT *et al.*, 1997) against open-access *Malus* EST databases. Data validation was performed by reverse tBLASTx and tBLASTn. The resulting alignments were filtered by a threshold e-value of $1e^{-25}$ and the hits were further

analyzed. Validated sequences were translated and protein (deduced amino acid) alignments were performed using ClustalX (THOMPSON *et al*., 1997).

Phylogenetic Analysis

Phylogenetic analyses were performed using distance and parsimony methods in the software PAUP* 4.0b10, using the software default parameters. Resampling bootstrap trees containing 1000 random samples were constructed using PSIGNFIT software.

Motif analysis and *in silico* characterization

Conserved motifs were further investigated by multiple alignment analyses using ClustalX and the MEME version 4.0 suite (BAILEY & ELKAN, 1994).

***In silico* gene expression analysis**

Qualitative gene expression profiling was performed by *in silico* analyses of the *Malus* EST databases using virtual northern blot analyses. The gene expression patterns among ESTs and libraries were obtained by hierarchical clustering based on Spearman Rank correlation matrix using Cluster v.2.11 software (EISEN *et al*., 1998). Graphic outputs were generated using Tree View v.1.6 (EISEN *et al*., 1998) and presented as a color scale.

Results and Discussion

The family of ethylene response factors in apple

Employing bioinformatic tools, we have established that the *Malus* AP2/ERF superfamily consists of 88 genes, clustered into three families; the ERF family (66 genes), the AP2 family (15) and the RAV family (7) (Table 1). Similar results were obtained by a work sequencing AP2-containing genes in apple (TACKEN *et al*., 2010). The conserved residues of AP2/ERF domain are present in all 66 ERF proteins in apple (Figure 1). The vast majority of the ERF-like sequences identified in apple shared one or more motifs outside the AP2/ERF domain with their *Arabidopsis* counterparts and were grouped in sub-groups as *Arabidopsis* proteins (Figure 1B). Apple sequences in group VII exhibit a *Malus*-exclusive conserved hydrophobic motif CMVII-9 (Figure 2).

Table 1. Summary of the AP2/ERF superfamily in *Arabidopsis thaliana* and *Malus* sp.

		Arabidopsis thaliana	*Malus sp.*
Classification	**Group**		**Number**
AP2 family		**18**	**15**
	double AP2/ERF domain	14	11
	single AP2/ERF domain	4	4
At4g13040		**1**	**0**
RAV family		**6**	**7**
ERF family		**122**	**66**
	groups I to IV	57	19
	group V to X	58	45
	groups VI-L and Xb-L	7	2
	Total	**147**	**88**

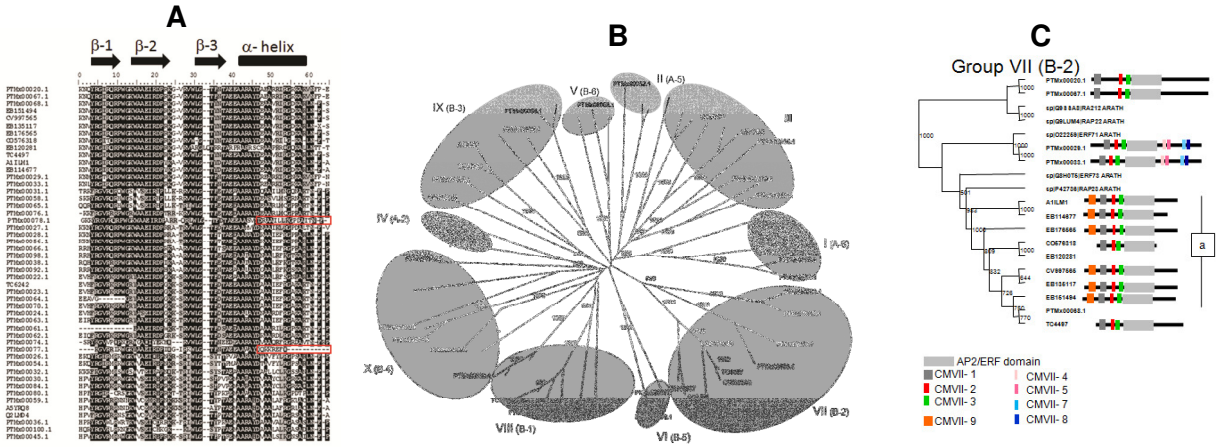

Figure 1. (**A**) Sequence alignment and structure prediction of the AP2/ERF domain of proteins from the ERF sub-family in *Malus* sp.. Black and light gray shading indicate identity and similarity. (**B**) and (**C**) Neighbor-joining tree for *Malus* and *Arabidopsis* and group VII sequences; respectively, aligned by ClustalX. Bootstrap values are above each branch.

In silico expression profiling of ERF and cell-wall enzyme genes in apple

Higher levels of expression of the *ERF* sequences from apple were observed in libraries derived from bud, root, xylem, phloem and fruit tissues (Figure 2). Differential expression libraries from pathogen-challenged tissues exhibited low frequency of transcripts derived from the investigated *ERF* sequences (Figure 2). Polygalacturonases were highly expressed in fruits, whereas β-galatosidase and PME were associated to leaf tissue (Figure2).

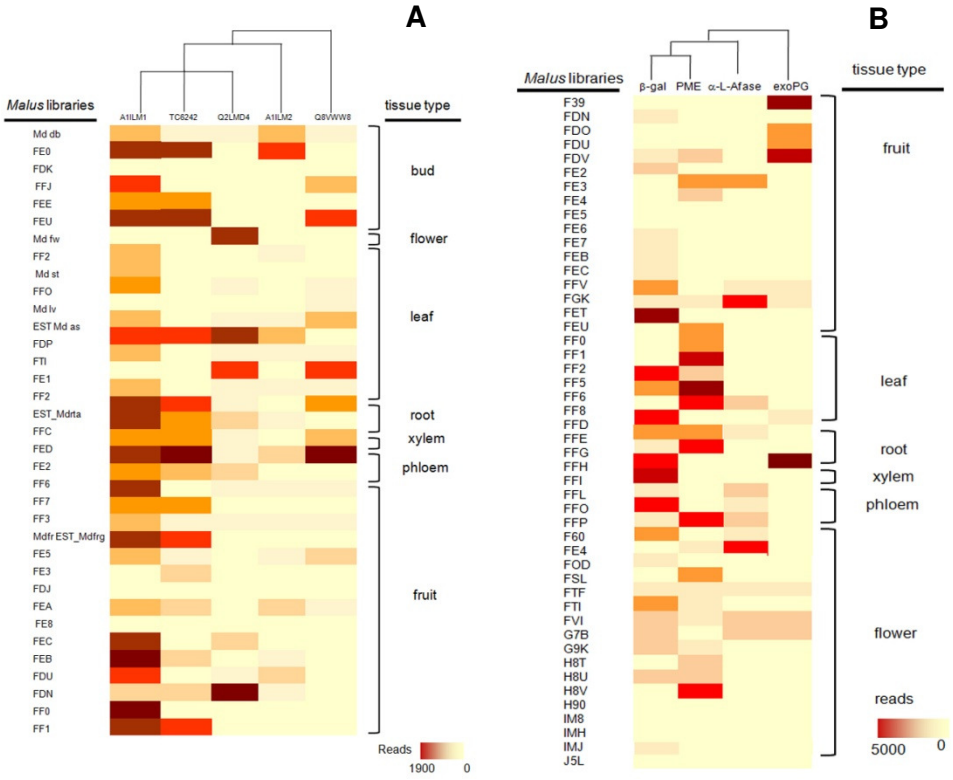

Figure 2. Expression profile of four ERF (**A**) and cell-wall enzyme (**B**) sequences in *Malus*. Hierarchical clustering of the expression patterns by *k*-means using Spearman Rank correlation is represented by tree.

Conclusions

In silico analyses allowed the identification of candidate ERF and cell-wall metabolism enzymes associated to ripening in apple. The tools are being used for *in vivo* studies.

Acknowledgements

Research is funded by Embrapa 02.07.05.001.00 Macroprograma 2 - Agrofuturo to CLG, CAPES-COFECUB 631/09.

References

Altschul, S.F.; Madden, L.; Schäffer, A.A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D.J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research, v. 25, p.3389-3402, 1997.

Bailey, T.L.; Elkan, C. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. In: Altman, R.; Brutlog, D.; Karp, P.; Lathrop, R.; Searls, D. (eds) Proc Second International Conference on Intelligent Systems for Molecular Biology. Menlo Park, CA: American Association for Artificial Intelligence Press, 1994, p 28–36.

Bapat, V.A.; Trivedi, P.K.; Ghosh, A.; Sane, V.A.; Ganapathi, T.R.; Nath, P. Ripening of fleshy fruit: molecular insight and the role of ethylene. Biotechnology Advances, v.28, p.94–107, 2010.

Eisen, M.B.; Spellman, P.T.; Brown, P.O.; Botstein, D. Cluster analysis and display of genome-wide expression patterns. Proceedings of the National Academy of Science USA v.95, p.14863-14868, 1998.

Nakano, T.; Suzuki, K.; Fujimara, T.; Shinshi, H. Genome-wide analysis of the ERF gene family in *Arabidopsis* and rice. Plant Physiology, v.140, p.411–432, 2006.

Stepanova, A.N.; Alonso, J.M. Ethylene signaling and response: where different regulatory modules meet. Current Opinion in Plant Biology, v.12, n.5, p.548-555, 2009.

Tacken, E.; Ireland, H.; Gunaseelan, K.; Karunairetnam, S.; Wang, D.; Schultz, K.; Bowen, J.; Atkinson, R.G.; Johnston, J.W.; Putterill, J.; Hellens, R.P.; Schaffer, R.J. The role of ethylene and cold temperature in the regulation of the apple *POLYGALACTURONASE1* gene and fruit softening. Plant Physiology, v.153, p. 294–305, 2010.

Thompson, J.D.; Gibson, T.J.; Plewniak, F.; Jeanmougin, F.; Higgins, D.G. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research, v.24, p.4876-4882, 1997.