Postharvest Biology and Technology 50 (2008) 117–124

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Isolation of genes potentially related to fruit quality by subtractive selective hybridization in tomato

D. Page [a,*], I. Marty [a], J.P. Bouchet [b], B. Gouble [a], M. Causse [b]

[a] INRA, UMR408, Sécurité et Qualité des Produits d'Origine Végétale, Université d'Avignon, F-84000 Avignon, France
[b] INRA, UR1052, Unité de Génétique et Amélioration des Fruits et Légumes, Domaine Saint-Maurice, BP94, F-84143 Montfavet Cedex, France

ARTICLE INFO

Article history:
Received 7 February 2008
Accepted 17 May 2008

Keywords:
Fruit ripening
Quantitative trait loci
qPCR
Solanum lycopersicum L.
Subtractive library

ABSTRACT

Improved understanding of the genetic and physiological control of quality traits could be helpful for identifying new technological or genetic targets to improve tomato fresh fruit quality and shelf life. This study aimed at screening for genes whose expression varied between lines genetically close but differing for fruit quality, using subtractive and selective hybridization (SSH). A set of 310 unigenes was isolated. The differential expression pattern of the SSH clones between LCx and Levovil, the two lines of the trial, was assessed by macroarray screening, and for 14 of them, by real-time PCR. Their putative functions were identified by BLAST comparison with public EST databases, and were classified on the basis of their function. Thirty nine percent of the unigenes corresponded to proteins which had never been isolated in fruit or with functions in fruit that were not clear or unknown. Among the others, proteins related to oxidative stress responses, calcium-binding proteins, a few cell-wall-related proteins, and several transcription regulators were identified. The SSH unigenes were then compared to the EST set of the tomato array Tom2 developed from public resources. The BLAST comparison revealed that 41% of the unigenes were not included in this set. This result revealed that our study emphasizes genes that would not have been considered with commercially available microarrays, and that constitute new targets for fruit quality control.

1. Introduction

Fleshy fruit are of primary importance for human food and health. However, as they naturally soften during their maturation, they remain fragile products that do not tolerate storage and transport well. However, their organoleptic quality, including the colour intensity, the sugar/acid balance or the release of aromatic compounds, is mainly established during the late stages of ripening. A further understanding of the genetic control of fruit quality traits should help in identifying breeding or biotechnological issues that favor the maintenance of quality traits without impairing, or even by improving, fruit shelf life.

Fruit softening involves a coordinated series of biochemical modifications of the polysaccharide components of the primary cell wall and middle lamella by a wide range of enzymes. Their *in vivo* knocking-out or over-expression in the model fruit tomato has a partial influence on the fruit softening and shelf life (Brummell and Harpster, 2001). However, up to now, genetic approaches only targeting these cell wall modifying enzymes have failed to fully control fruit softening. All these enzymes follow a ripening-related scenario, involving a complex regulation network (Lelievre et al., 1997; Trainotti et al., 2006). Targeting regulators that coordinate the ripening process has been more successful. The first regulator studied was ethylene, which was for a long time considered as the trigger for fruit maturation (Oeller et al., 1991). Mutations altering its biosynthesis or its perception by tissues have a large impact on maturation (Lelievre et al., 1997). Genomic studies revealed that a large proportion of the ripening-related genes (roughly 40%) depend on ethylene (Alba et al., 2005). These molecular studies also revealed that ethylene is most likely to be an enhancer acting downstream of a chain of interrelated regulators (Adams-Phillips et al., 2004). The molecular characterization of maturation-affected mutants like the *ripening-inhibitor* [*rin*] and the *Colourless non-ripening* [*Cnr*] mutants confirmed these hypotheses. The *rin* + mutant, for which all the ripening phenomena are inhibited, is affected for a MADS-box gene, a transcription factor acting upstream of the ethylene ripening control (Vrebalov et al., 2002). The *Cnr* mutant exhibits colourless and mealy pericarp due to a substantial loss of cell-to-cell adhesion, and results from an epigenetic change in the promoter of a squamosa promoter-binding protein like (SBP-box) gene (Manning et al., 2006). The

* Corresponding author. Tel.: +33 4 32 72 24 91; fax: +33 4 32 72 24 92.
E-mail address: dpage@avignon.inra.fr (D. Page).

0925-5214/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.postharvbio.2008.05.017

Rin and *Cnr* genes have regulation roles with distinct but major and related effects. Therefore, they were likely to be isolated by a mutant approach. This is less evident for more transient and minor regulators. However, these kinds of regulatory genes correspond more to our target, as they are likely to control only some aspects of fruit development without fully disrupting the maturation process.

Non-targeted genomic screenings have led to the delivery of well-organised and interrelated EST databases. The isolated genes cover a wide range of functions, including hormone response-related genes as well as transcription factors (Giovannoni, 2004). However, such descriptions did not allow identification of regulators particularly related to fruit softening. These collections were tentatively narrowed by digital expression analysis and comparative genomics comparing fleshy fruit ESTs (grape, tomato) to *Arabidopsis thaliana*, leading to the identification of a set of 24 fruit-specific ESTs (Fei et al., 2004). Although this set is interestingly reduced and a quarter of these genes were transcription factors, their relations to fruit texture were not further assessed.

In this study, we used genetically close lines developed on the basis of genome regions involved in fruit quality control to focus our genomic screening on fruit quality control. The lines came from a cross between Levovil, a line with 120–150 g fruit with an ordinary taste and low texture quality, and Cervil, a cherry tomato with 7 g fruit and good tasting. Quality traits were measured in the progeny by sensory analysis (Causse et al., 2001) or instrumental measurements (Saliba-Colombani et al., 2001). The combination of genetic and quality measurements allowed the identification of five regions (QTLs) on four chromosomes involved in fruit quality control. Marker assisted breeding was then performed to produce genetically close lines varying for these QTLs (Lecomte et al., 2004). We used LCx, a line carrying 30% of the Cervil genome, with smaller fruit than Levovil, and differing for the five major QTL regions, which were in contrast with Levovil for most of the measured sensory attributes (Causse et al., 2007).

We used the contrast LCx/Levovil to build a fruit quality-related EST bank. Genes for which the expression differed in fruit of these two lines were isolated using subtractive and selective hybridization (SSH) of mRNA (Diatchenko et al., 1996). In our case, SSH presented not only the interest of isolating contrasted genes, but also of normalising the mRNA amounts: SSH libraries are enriched in low expression genes and they are limited in their content of highly expressed genes (Diatchenko et al., 1996). Differential expression of SSH clones between Levovil and LCx were globally verified by macroarrays, and in detail by real-time PCR for a set of selected genes.

2. Materials and methods

2.1. Plant material and sampling

Tomato (*Solanum lycopersicon* L.) lines used were Levovil, the recurrent parent, and LCx derived by marker-assisted selection from a cross between Levovil and Cervil, this last line being the line carrying the beneficial alleles (Lecomte et al., 2004). LCx had the Cervil alleles at five QTL regions carried on chromosomes 1, 2, 4 and 9 (2 regions). Lines were grown in 2002, during spring in a heated glasshouse, in Avignon (south of France). Each line was represented by a single plot of six plants, grown in soil. Thirty fruit per genotype were picked during the fruit production period. The fruit were harvested at breaker (B) and red ripe (RR) stages.

A fruit-by-fruit evaluation was performed on the 30 fruit for the physical traits. Based on the individual results, a batch of homogenous fruit was constituted for each stage and line by reassembling fruit exhibiting firmness within the confidence interval ($p < 0.05$) of the mean calculated from the 30 fruit. Batches of frozen powder were then constituted by grinding one quarter of each retained fruit and aliquots were used for mRNA extraction.

2.2. Physical, physiological and chemical measurements

Fruit texture was assessed with a Penelaup® (Sérisud, Montpellier, France): this apparatus registered force/deformation curves by measuring the reaction force in response to an increasing mechanical constraint applied to the fruit by different probes supported by a motorised arm. For the compression test, a 5-cm diameter probe was used to compress each fruit until obtaining a deformation corresponding to 3% of the fruit diameter. The probe speed was 20 cm min^{-1}. The firmness was then calculated as the pressure necessary to obtain this deformation. Firmness is an overall estimation of the fruit resistance including skin resistance and flesh firmness (Grotte et al., 2001). For the puncture test, the probe was a 2-mm cylinder with a flat end and the constraint was applied to the fruit until the probe passed through the skin. The probe speed was 10 cm min^{-1}. The elasticity and the deformation were calculated as, respectively, the force and the distance required to pass through the skin. Chemical variables were the average of 7 batches of 10 fruit each corresponding to a 2-year trial described in Chaïb et al. (2006). The measurements were soluble solids content, titratable acidity and sugar content and were performed as previously described (Saliba-Colombani et al., 2001).

2.3. mRNA isolation

RNA was extracted from 5 g aliquots of frozen fruit powder according to the method described by Chang et al. (1993), except that the final RNA precipitation was performed 2.5 h at room temperature in a 3 M sodium acetate solution. RNA concentration was measured by spectrometry and the extraction was considered as successful when the yield was higher than 20 μg of RNA per gram of fruit powder. Poly(A) + mRNA were then purified using the poly-ATract isolation system (Promega, Madison, USA). In order to reach the requirement for starting with the SSH kit, the mRNA solutions were concentrated to 500 ng/μL under vacuum.

2.4. SSH library construction

Four libraries were built: two at each of the two stages of development (breaker and red ripe) using the PCR Select™ cDNA subtraction kit (Clontech, Palo Alto, CA). For the two forward libraries, i.e. libraries enriched with genes mostly expressed in Levovil (named Levovil-enriched libraries), Levovil mRNAs were used as tester, and the LCx mRNAs as driver. For the reverse libraries, i.e. libraries enriched with genes mostly expressed in LCx (named LCx-enriched libraries), LCx mRNAs were used as tester and Levovil mRNAs as driver. The SSH was performed according to the manufacturer's recommendations, except for the second hybridization where the ratio tester:driver was set at 1:10 instead of 1:3 in order to amplify the subtraction effect. All PCR and hybridization steps were performed on a PerkinElmer (Waltham, USA) 2400 thermal cycler. The adaptator-ligation efficiency and the subtraction efficiency tests were performed as described using PCR primers designed on the LE-ACC2 tomato gene (GB:X59139) as internal standard. Forward primer was ACC2-F 5′-TTAAAAGGGAAGAATTTAATT-3′ and reverse primer was ACC2-R 5′-TTATTTTCTTTCATCAGTTTGCACA-3′. The subtracted cDNAs were then cloned into the pCR® 4-TOPO® Vector (Invitrogen, Carlsbad, USA).