Effect of the *Colorless non-ripening* Mutation on Cell Wall Biochemistry and Gene Expression during Tomato Fruit Development and Ripening[1[w]]

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The *Colorless non-ripening* (*Cnr*) mutation in tomato (*Solanum lycopersicum*) results in mature fruits with colorless pericarp tissue showing an excessive loss of cell adhesion (A.J. Thompson, M. Tor, C.S. Barry, J. Vrebalov, C. Orfila, M.C. Jarvis, J.J. Giovannoni, D. Grierson, G.B. Seymour [1999] Plant Physiol 120: 383–390). This pleiotropic mutation is an important tool for investigating the biochemical and molecular basis of cell separation during ripening. This study reports on the changes in enzyme activity associated with cell wall disassembly in *Cnr* and the effect of the mutation on the program of ripening-related gene expression. Real-time PCR and biochemical analysis demonstrated that the expression and activity of a range of cell wall-degrading enzymes was altered in *Cnr* during both development and ripening. These enzymes included polygalacturonase, pectinesterase (PE), galactanase, and xyloglucan endotransglycosylase. In the case of PE, the protein product of the ripening-related isoform PE2 was not detected in the mutant. In contrast with wild type, *Cnr* fruits were rich in basic chitinase and peroxidase activity. A microarray and differential screen were used to profile the pattern of gene expression in wild-type and *Cnr* fruits. They revealed a picture of the gene expression in the mutant that was largely consistent with the real-time PCR and biochemical experiments. Additionally, these experiments demonstrated that the *Cnr* mutation had a profound effect on many aspects of ripening-related gene expression. This included a severe reduction in the expression of ripening-related genes in mature fruits and indications of premature expression of some of these genes in immature fruits. The program of gene expression in *Cnr* resembles to some degree that found in dehiscence or abscission zones. We speculate that there is a link between events controlling cell separation in tomato, a fleshy fruit, and those involved in the formation of dehiscence zones in dry fruits.

Colorless non-ripening (*Cnr*) is a pleiotropic dominant mutation of tomato (*Solanum lycopersicum*) that results in fruits with a white pericarp displaying much reduced cell-to-cell adhesion (Thompson et al., 1999; Fraser et al., 2001). The *Cnr* locus has been mapped to the middle of the long arm of chromosome 2 and is currently the subject of a map-based cloning exercise (Tor et al., 2002). We have recently isolated and sequenced a region of tomato chromosome 2 that cosegregates with the *Cnr* locus and are testing a candidate gene at this locus (K. Manning, J.J. Giovannoni, and G.B. Seymour, unpublished data).

The loss of cell adhesion in *Cnr* appears to be due principally to modifications in cell wall structure. Sections of *Cnr* pericarp tissue show obvious changes in comparison with wild-type fruits, including larger intercellular spaces and thinner cell walls in ripe fruits (Orfila et al., 2001). Mechanical tests on pericarp tissue have revealed that the force required for cell wall failure is greater in *Cnr*, while tests on cell wall preparations showed that the cell walls were less able to swell in water in comparison to wild type. These observations indicate that *Cnr* has stronger, less swollen, less adherent cell walls than wild-type fruits at the ripe stage (Orfila et al., 2001).

The biochemical changes that account for the differences in the physical properties of *Cnr* and wild-type cell walls have been investigated. Cell adhesion in plants is known to involve calcium cross-linking of deesterified regions of adjacent pectic polysaccharides. Electron energy-loss spectroscopy has been used to show that *Cnr* cell walls have a reduced calcium-binding capacity, and this reflects modifications in the structure of the pectic polysaccharides. These modifications include the likely absence of long stretches of deesterified pectin for calcium binding, disrupted deposition of (1-5)-α-L-arabinan, and significantly reduced solubility of pectic polysaccharides from the *Cnr* cell walls (Orfila et al., 2001, 2002). The effects on cell wall biochemistry are not confined to the

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pectic polysaccharides alone. A cellulose-associated microdomain involving a tight association of arabinosyl- and galactosyl-containing pectic polysaccharides and cellulose microfibrils is present in both wild-type and *Cnr* fruits but undergoes significant degradation in wild type, and this may influence the subsequent porosity of the cell walls and other properties relating to cell wall degradation (Orfila et al., 2002).

These alterations in *Cnr* cell walls are likely to be the cause of the enhanced cell separation in *Cnr* pericarp and almost certainly reflect a different program of gene expression and cell wall-degrading activities in the mutant. Only a limited amount of information is available on changes in gene expression linked to the altered phenotype in *Cnr* fruits. Initial studies indicated that a wide range of ripening-related genes might be affected (Thompson et al., 1999). Detailed information has not been published on the altered pattern of gene expression in the mutant, and such information will be critical to make the connections between the regulatory networks controlling normal ripening and those perturbed in *Cnr*. Genes controlling cell separation in plants have remained elusive, and *Cnr* is among the few documented examples where a mutation has substantially altered this process. This mutant provides a route for exploring the regulation of fruit ripening and finding the genes involved in the control of cell-to-cell adhesion.

In this article, we describe the effects of the *Cnr* mutation on changes in cell wall biochemistry and gene expression during tomato fruit development and ripening that may explain the enhanced cell separation phenotype in the mutant. Furthermore, the *Cnr* mutation resulted in a pattern of gene expression reminiscent, to some degree, of that found in abscission or dehiscence zones, providing potential new insights into links between mechanisms controlling aspects of cell separation in fleshy and dry dehiscent fruits.

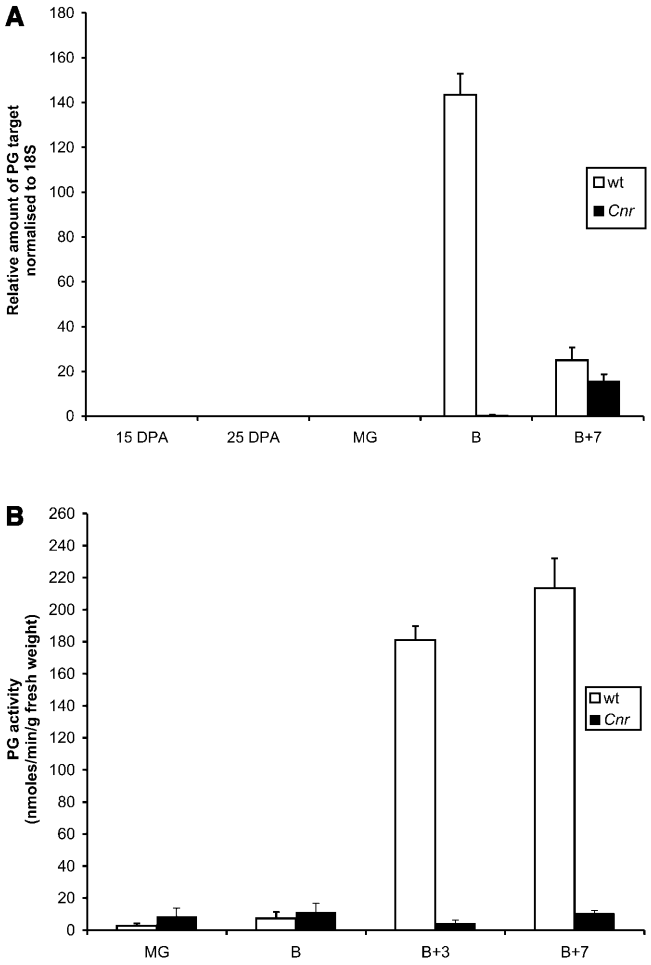

Figure 1. Real-time PCR expression data (A) and activity measurements (B) of PG (accession no. X04583) during the development and ripening of wild-type and *Cnr* fruits.

RESULTS

The Expression and Activity of Enzymes Associated with Cell Wall Disassembly

Polygalacturonase

Polygalacturonase (PG) gene expression (PG2a, accession no. X04583; Grierson et al., 1986) was quantified by real-time PCR with a comparative C_T method, using a TaqMan (Sigma Genesis, Dorset, UK) probe; the results are shown in Figure 1A. In wild-type fruit, there was no detectable PG expression at 15 DPA; very low levels were detectable at 25 DPA and mature green (MG). PG expression was highest at the breaker (B) stage of ripening, but by breaker + 7 d (B + 7) the amount of PG expression in the wild type had decreased dramatically. In *Cnr* no PG expression was detected at 15 DPA, but, as in wild type, very low levels were found at 25 DPA and MG. PG expression was also detectable at B, but this was at only 0.3% of that detected in wild-type fruit at this stage of ripening. In contrast with wild type, the expression of PG in *Cnr* appears to increase during ripening such that levels at B + 7 are higher than at B and are approaching those found in wild-type fruit at this time. Changes in the level of PG activity were measured during ripening at MG, B, breaker + 3 d (B + 3), and B + 7 in wild-type and *Cnr* fruits. At B + 3 and B + 7, the activity was significantly lower in *Cnr* fruit pericarp compared to wild type (Fig. 1B). PG activity increased dramatically in wild-type fruit shortly after the B stage, whereas in *Cnr* fruit, activity was only ever detected at very low levels (Fig. 1B).

Pectinesterase

Real-time PCR quantification of the ripening-related pectinesterase (PE) isoform 2 (PE2; accession no. X07910) revealed that in wild-type fruit, expression is low at 15 DPA, peaks at 25 DPA, and then decreases steadily through ripening. In *Cnr* fruit at 15 DPA, PE2 mRNA level is significantly higher (Mann-Whitney *U*

test, significance level 0.5%) than in the wild type. However, no increase in expression follows, and by 25 DPA, expression of PE2 is significantly lower in *Cnr* than in wild type. Then similarly to wild type, PE2 expression in *Cnr* decreases during further development and ripening (Fig. 2A). The level of PE activity was measured during development and ripening. The activity of PE (Fig. 2B) increased significantly during normal ripening, but levels remained low throughout *Cnr* fruit development.

Changes in PE activity were explored further by isoform profiling using heparin chromatography. Extracts from ripe wild-type tomato produced a profile with three distinct isoforms that correspond with those described in previously published work (Simons and Tucker, 1999): PE2 (fractions 26–36), PE3 (fractions 37–41), and PE1 (fractions 45–54; Fig. 2C). Profiles of *Cnr* ripe extracts revealed the absence of the fruit-specific isoform PE2, which in wild-type fruits accounts for approximately 70% of the total activity

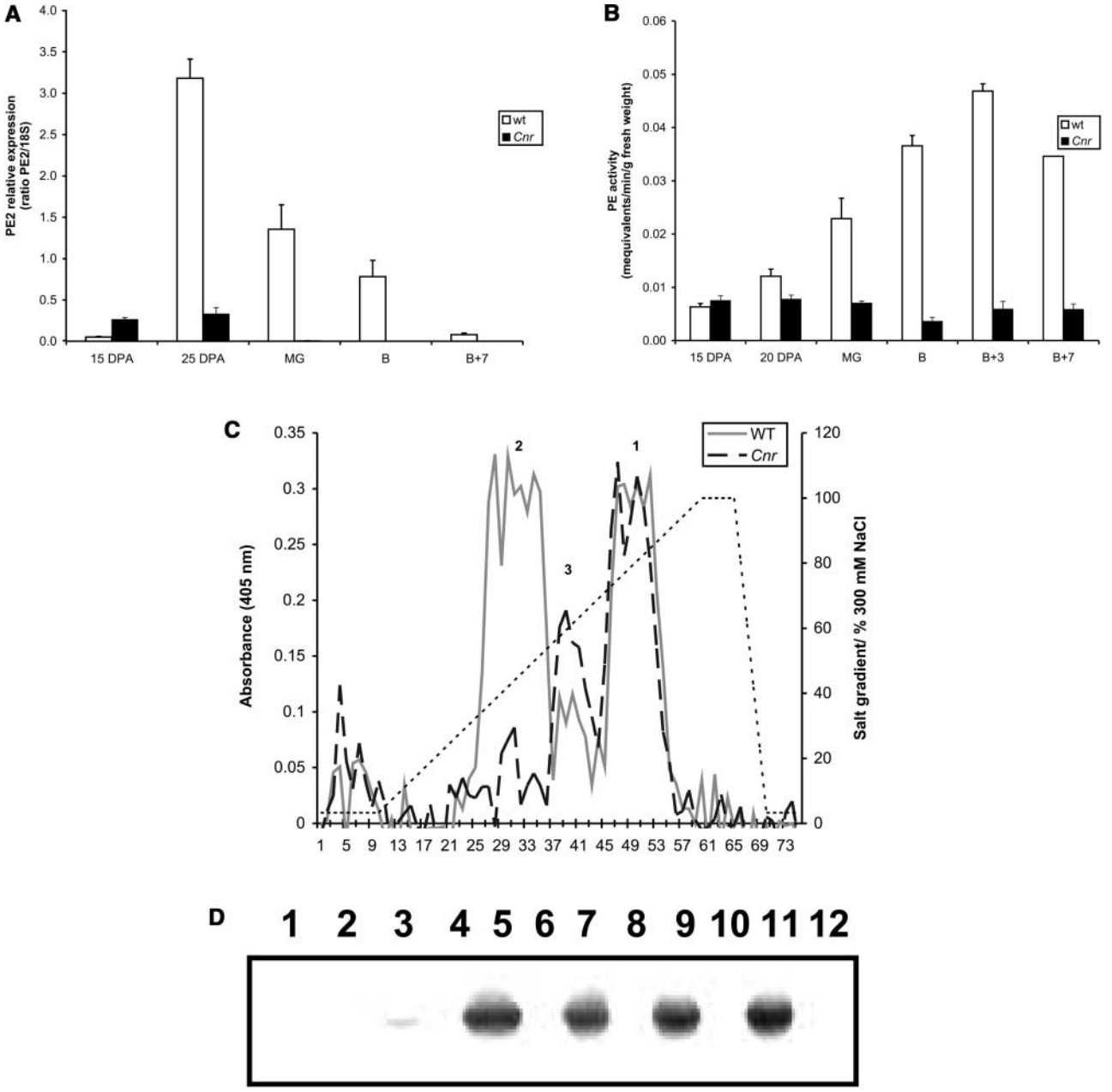

Figure 2. A and B, Real-time PCR expression data for PE (accession no. U70675; A) and total PE activity measurements (B) during the development and ripening of wild-type and *Cnr* fruits. C, PE isoforms , marked 1, 2, and 3 on chromatogram, in wild-type and *Cnr* fruit at B + 7. D, Immunodetection of PE2 using a polyclonal antibody; lanes 1, 3, 5, 7, 9, and 11, WT 15 DPA, 20 DPA, MG, B, B + 3, and B + 7; lanes 2, 4, 6, 8, and 12, *Cnr* 15 DPA, 20 DPA, MG, B, B + 3, and B + 7.

(Tucker et al., 1982). In a western blot of crude protein extracts from wild-type and *Cnr* pericarp, a polyclonal antibody for PE2 detected this protein at all stages after 20 DPA in normal fruit, but not in *Cnr* fruit at any stage of development (Fig. 2D).

Basic Chitinase

Analysis of protein profiles from developing wild-type and *Cnr* pericarp by SDS-PAGE revealed a number of distinct differences during both development and ripening (Fig. 3A). Two prominent bands, with molecular masses of around 28 and 26 kD which were exclusive to the *Cnr* profile at the MG through to ripe stages, were cut out after transfer to a polyvinylidene difluoride membrane and N-terminally sequenced. Homology searches using the PAM30 matrix within the BLASTp algorithm (Altschul et al., 1997) revealed significant similarities to the N-terminal chitin-binding domain of a 30-kD and a 32-kD class I basic chitinase (Joosten et al., 1995), respectively. The 30-kD basic class I chitinase protein had the same sequence as a basic chitinase identified from the differential screen (Table I).

An up-regulation of basic chitinase gene expression (The Institute for Genomic Research [TIGR] Tomato Gene Index, TC115944) was demonstrated by real-time PCR in both wild type and *Cnr* at the MG stage; however, the up-regulation seen in *Cnr* from 25 DPA to MG was considerably more dramatic (Fig. 3B). In *Cnr* the expression level then stayed at a high level until B stage, after which it decreased significantly. In the wild type, mRNA abundance was highest at MG and decreased slowly throughout ripening.

β-Galactanase, Xyloglucan Endotransglycosylase, and Peroxidase

Enzyme measurements also revealed an effect of the mutation on β-galactanase (β-gal) and xyloglucan endotransglycosylase (XET) activity during ripening at MG, B, B + 3, and B + 7 in wild-type and *Cnr* fruits. At B + 7, the activity of both enzymes was significantly lower in *Cnr* fruit pericarp compared to wild type (Fig. 4, A and B). The increase in β-gal observed during normal ripening was not seen in *Cnr*, where β-gal activity stayed at a constant but low level (Fig. 4A). There was a trend of decreasing XET activity during ripening in both wild-type and *Cnr* fruit, although lower levels were observed in *Cnr* from all stages from B to ripe (Fig. 4B). The activities of β-gal and XET were similar in wild type and *Cnr* at the MG stage. For all the measurements of enzyme activity reported in this paper, the same trends were apparent whether enzyme activities were expressed on a per-milligram-protein or on a tissue-fresh-weight basis.

Figure 4C shows nitrocellulose tissue prints of both wild-type and *Cnr* mature fruits stained for peroxidase activity. Peroxidase activity was shown to be concentrated in the exocarp (skin) and the radial vascular network running through the central regions of the pericarp in fruits from wild type. By contrast, mature fruits from the *Cnr* mutant not only showed a greater intensity of peroxidase staining in comparison to wild type, but also had significantly more staining in other tissues of the fruit. Peroxidase staining was observed in the columella and radial pericarp wall (septa) in the *Cnr* mutant but was absent from these regions in wild type. Preliminary experiments to assess the extent of soluble and wall-bound peroxidase isoenzymes revealed a wall-bound fraction in *Cnr* fruits from 20 DPA through to B + 7. No similar wall-bound fraction was apparent in wild-type fruits (data not shown).

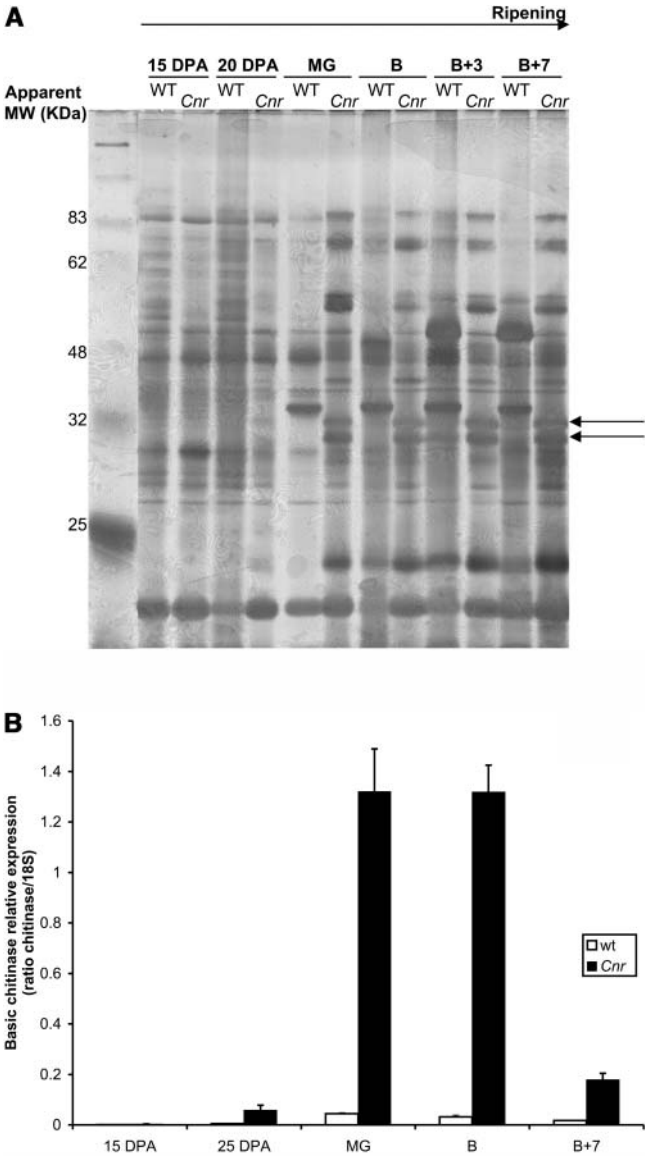

Figure 3. A, SDS-PAGE profiles of cell wall-bound and soluble proteins from developing and ripening pericarp of wild type and *Cnr*. Arrows indicate positions of bands identified as basic chitinases after blotting and excision for N-terminal sequencing. Proteins bands were revealed by silver staining. B, Real-time PCR determination of expression of basic chitinase (accession no. TC115944) during the development and ripening of wild-type and *Cnr* fruits.

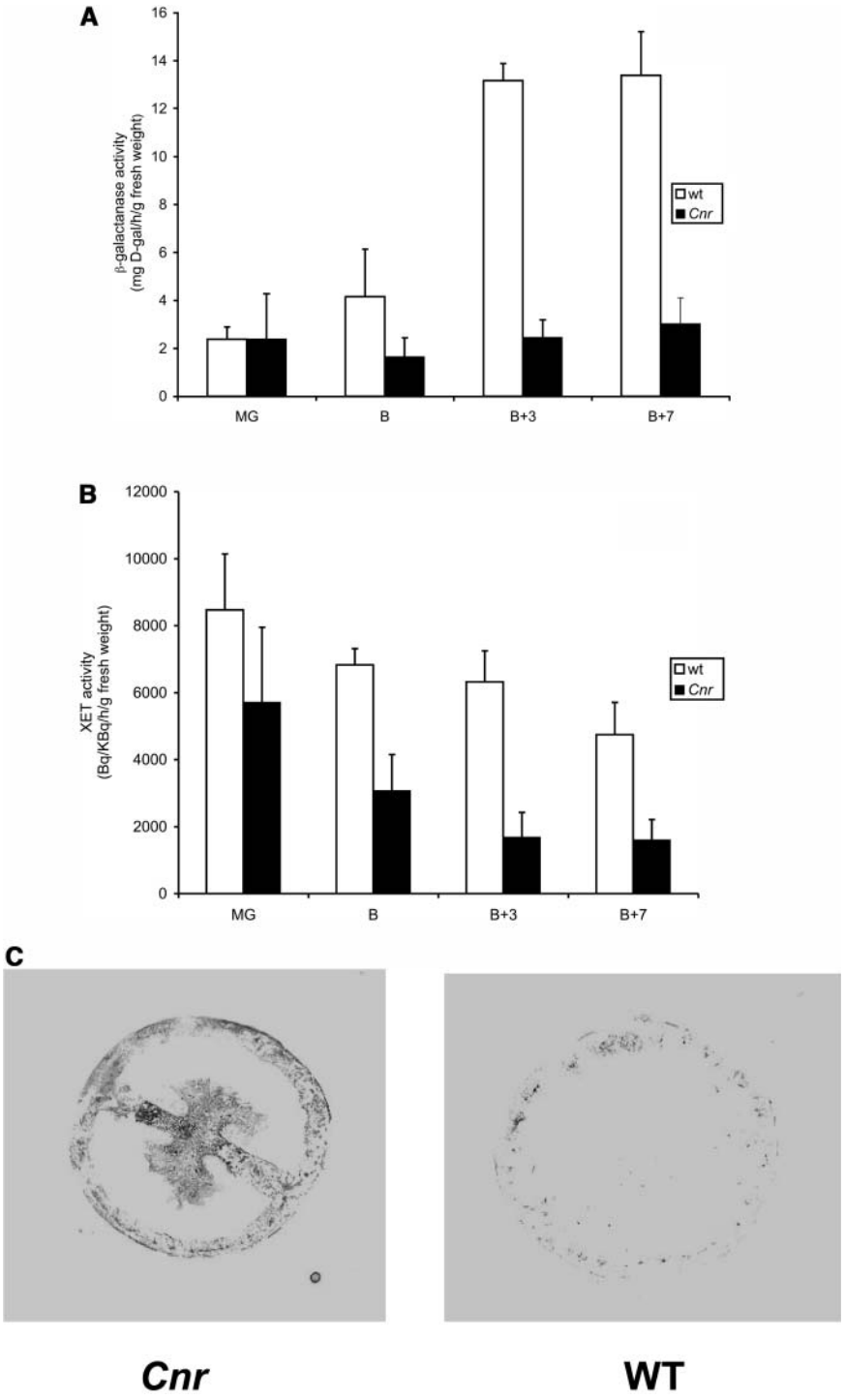

Figure 4. A and B, Determination of (A) β-gal and (B) XET activity during the development and ripening of wild-type and *Cnr* fruits. C, Peroxidase activity in tissue prints of wild-type and *Cnr* fruits at B + 7.

Effect of the *Cnr* Mutation on the Program of Gene Expression during Fruit Development and Ripening

Microarray Analysis

To evaluate the extent of gene expression changes in the *Cnr* mutant, microarray hybridizations were performed. A total of four samples (wild-type 15 DPA, wild-type B + 7, *Cnr* 15 DPA, *Cnr* B + 7) were compared against each other, through the use of a common reference sample in each dual hybridization experiment (see ''Materials and Methods''). There were a significant number of differences between the samples. After selecting for clones which showed a 2-fold or more difference between any of the four samples and filtering out redundant clones, a total of 477 unique genes were clustered into groups according

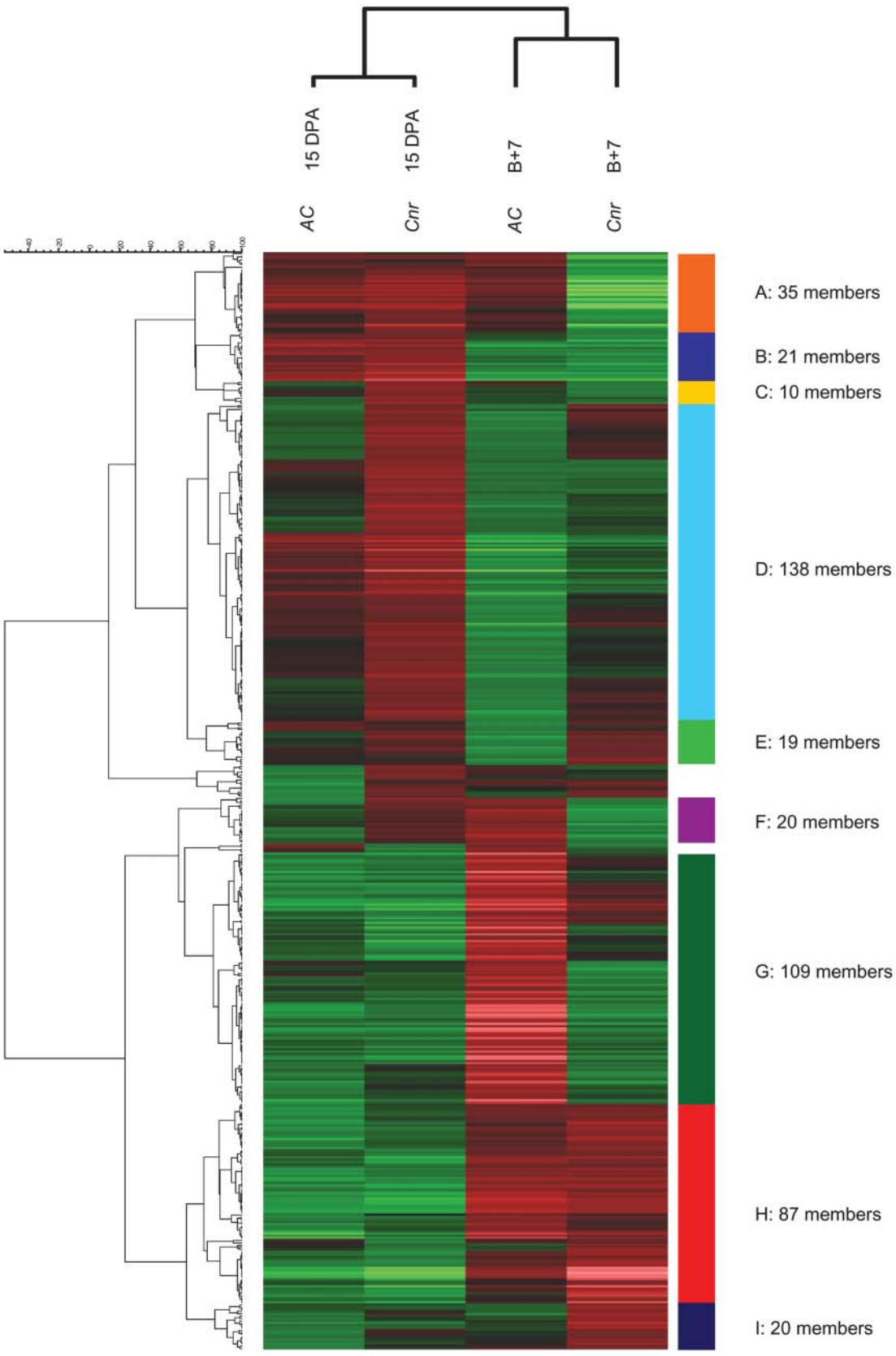

Figure 5. Hierarchical clustering of genes and samples of wild-type and *Cnr* fruit. After selection of nonredundant clones that showed a differential expression (2-fold or more) between any of the 4 samples compared (wild-type 15 DPA; *Cnr* 15 DPA; wild-type B + 7; *Cnr* B + 7), 477 clones and the 4 samples were clustered into groups using the Pearson clustering algorithm. Genes showing more than 75% similarity in expression over the four samples are shown as a group (marked A–I).

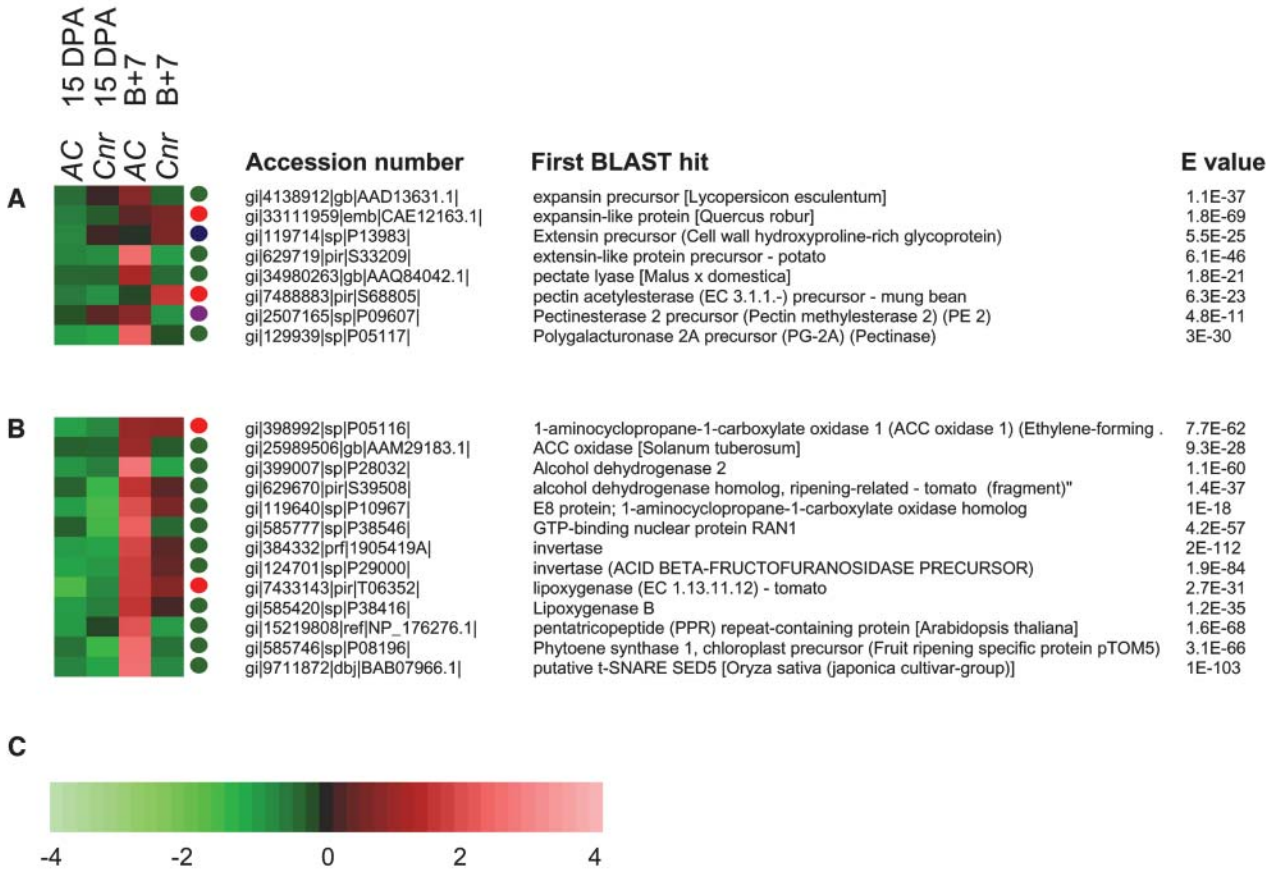

Figure 6. Gene expression patterns of selected ripening-related genes. Gene expression patterns and homology of genes involved in cell wall disassembly (A) and other ripening-related processes (B). The standardized expression level of each gene over the four samples (wild-type 15DPA, *Cnr* 15 DPA, wild-type B + 7, and *Cnr* B + 7, respectively) is visualized as color bars. The color scale ranges from −4 to +4 on a ^{2}Log scale (C). For each clone, the accession number of the most homologous (deduced) protein in the *NR* database (BLAST-X), the ID assignment of the first BLAST hit, and the corresponding *E*-value are shown.

to expression behavior using the Pearson clustering algorithm (Fig. 5). The complete data set for Figure 5 is presented as supplemental material (available at www.plantphysiol.org). Details of some of the most informative data extracted from the microarray are presented below.

Effects of the Mutation on the Overall Pattern of Gene Expression

Of all the nonredundant, differentially expressed genes, only a small proportion (22%) showed a similar expression pattern of up- or down-regulation from 15 DPA to B + 7 in both wild-type and *Cnr* fruits (groups B and H). Group B (4%) included genes expressed most highly in immature green fruit, such as ribulose bisphosphate carboxylase, and group H (18%), ripening-associated genes such as 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase). The genes with altered expression levels in *Cnr* fruits consisted of two large groups (group D and G) and several smaller groups. The largest group (group D; 30%) was composed of green fruit-specific and constitutive genes that were up-regulated in *Cnr*, in particular in immature green *Cnr* fruit. Several of these encode proteins normally associated with stress responses, such as a protease inhibitor and stress-responsive protein. Group G (24%) was composed of ripening-related genes that were much more highly expressed in wild-type ripe than in *Cnr* ripe or wild-type unripe fruits, and these included PG and phytoene synthase. In addition to these two large groups, several smaller groups showing varying expression patterns in wild type and *Cnr* were also apparent (Fig. 5). In group A, genes showed high levels of expression in both immature and ripe wild-type fruits, but were down-regulated in ripe *Cnr* fruit tissues. Group C consisted of constitutive genes that were strongly up-regulated in *Cnr* unripe fruits and down-regulated in *Cnr* ripe fruits. In group E, green fruit-specific genes with elevated expression levels in ripe *Cnr* fruits were clustered. As in group D, this group contained several stress-related genes. Group F consisted of ripening-related genes (such as, for example, PE1 and PE2) that were up-regulated in immature green *Cnr* fruits, up to levels as high as in wild-type ripe fruits, but were

down-regulated in ripe *Cnr* fruits. Finally, group I consisted of constitutive genes with increased expression levels in ripe *Cnr* fruits.

Genes Associated with Cell Wall Disassembly and Other Ripening Events

The microarray experiments were generally consistent with and added to our information on changes in genes involved in modulating cell wall structure. Genes encoding the cell wall enzymes PG and PE2 were clearly suppressed in *Cnr* along with transcripts for pectate lyase, another pectin-degrading enzyme, and a possible extensin-like protein. However, in *Cnr* the level of transcripts for both an expansin-like gene and pectin acetylesterase were enhanced above those found in wild-type tissues (Fig. 6A). Genes with roles in ripening other than cell wall degradation were also profoundly affected by the mutation, including phytoene synthase, E8, the ethylene signal transduction GTP-binding protein RAN1, a likely plastid pentatricopeptide (PPR) repeat-containing protein, acid invertase, alcohol dehydrogenase, lipoxygenase, an ACC oxidase homolog, and a putative t-SNARE SED5 gene (Fig. 6B).

Differential Screen

A differential screen was performed to identify genes that might not be present on the microarray and were up-regulated in *Cnr*. Of the 80 clones that hybridized with the *Cnr* probe, 55 contained inserts that could be amplified by PCR. Sequencing of these amplicons and the appropriate BLAST searches revealed a range of putative gene functions (Table I). The most frequent clones from this screen were those with homologies to basic chitinase (29%; 14 out of 48 sequenced clones). The basic chitinases were represented by two different genes that were named chitinase 1 (11 clones; TC115816) and chitinase 2 (3 clones; TC115944). The second most frequently appearing group of clones in this screen had homologies to genes encoding pathogenesis-related (PR) proteins. These made up 23% of the total clones: 9 homologous to the gene for PR-protein P1 (P14), 1 to P23, and 1 to P24. The remaining clones all had different sequences and homologies and included a metallothionein-like protein and ubiquitin-conjugating enzyme (Table I).

Changes in the Expression of Regulatory Factors in Cnr Fruits

The gene at the *rin* locus has been shown to be essential for ripening and is a MADS box transcription factor (Vrebalov et al., 2002). Preliminary experiments had indicated that MADS box transcription factor gene expression was altered in the *Cnr* mutation (M. Tör, L. Harrison, and G.B. Seymour, unpublished data). The microarray experiments confirmed that the expression of a number of regulatory genes was affected by the presence of the *Cnr* mutation, including those of the MADS box class. The expression of two genes of this class was investigated in greater detail by real-time PCR. The expression of *LeMADS-RIN* was detected at low levels in both 25-DPA wild-type and *Cnr* fruits. A similar level of expression was observed at B; however, at B + 7, *LeMADS-RIN* mRNA levels were significantly higher in the mutant (Fig. 7A). *TDR4* was found to be highly up-regulated during normal ripening but substantially suppressed in *Cnr* from 25 DPA (Fig. 7B).

DISCUSSION

One of the most apparent features of the *Cnr* mutation is a loss of pericarp cell adhesion in mature fruits. This altered cell separation phenotype in *Cnr* is likely to be linked with changes in cell wall biochemistry brought about through an altered program of cell wall-related gene expression. We found that the activities of a variety of pectin-degrading enzymes were reduced in the mutant in both developing and ripening fruits. Several genes are responsible for PE activity during normal ripening. PE2 contributes most of the activity, and its gene is expressed during fruit development and ripening in wild type. Real-time PCR, enzyme analysis, western blotting, and microarray experiments all consistently indicated a reduced *PE2* gene expression and PE2 protein in *Cnr* fruits. Antisense experiments with PE2 have demonstrated that a reduction in the levels of this isoform during ripening significantly retards pectin deesterification in the fruit pericarp (Hall et al., 1993). Changes in pectin esterification almost certainly contribute to significant reduction in the calcium-binding capacity of the pectic polysaccharides observed in the mutant (Orfila et al., 2001). However, these changes can only partly be responsible for the altered texture phenotype in *Cnr* because transgenic experiments to suppress PE activity in tomato fruit (Tieman and Handa, 1994) did not affect fruit softening. In this study, a variety of cell wall-degrading enzymes, along with PE, showed lower activity in *Cnr*, including PG, β-gal, and XET, and the microarray experiment also indicated a reduction in pectate lyase expression in the mutant. We have already shown that *Cnr* cell walls are stronger and less swollen than wild type while still being less adherent (Orfila et al., 2001). Reduced activities of PG, β-gal, XET, and pectate lyase could help maintain a strong cell wall, which in combination with weak calcium binding in the middle lamella could encourage cell separation rather than bursting of cells on mechanical manipulation.

Chitinases appear to be especially prevalent in *Cnr* fruits and might contribute to cell separation as well as to protecting the tissues from pathogen invasion. The carrot (*Daucus carota*) EP3-3 chitinase gene identified and characterized by Passarinho et al. (2001) has been shown to cleave arabinogalactan proteins (AGPs) in

Table I. *TIGR database homologies of sequenced clones from a differential screen of a Cnr MG cDNA library*

These were highly expressed in *Cnr* MG but not in wild-type MG fruit.

Cnr Clone No.		DNA Sequence Similarity	*E*-Value	Gene ID Assignment
		%		
1	TC115816	99	1.8e−97	Chitinase
2	TC115911	98	9.5e−133	PR protein; P1(P14) protein; PR protein P6
3	TC115816	99	1.8e−97	Chitinase
4	BI207227	95	4.3e−86	60S ribosomal protein L30
5	TC115816	92	1.0e−86	Chitinase
6	TC115816	95	4.2e−83	Chitinase
7	TC119839	95	7.4e−83	Phosphatidic acid phosphatase α
8	TC124117	95	1.9e−45	Calreticulin
9	TC116966	99	8.9e−89	26S proteasome p55 protein-like
10	TC115816	100	5.8e−90	Chitinase
11	TC124921	98	3.4e−34	Possible apospory-associated protein C
12	TC116424	89	1.4e−78	Histone H1
13	TC116545	87	2.0e−120	Osmotin-like homolog; PR protein P23
14	TC115911	100	6.7e−74	PR protein; P1(P14) protein; PR protein P6
15	TC126217	97	3.8e−55	RNA-binding protein RZ-1
16	TC130480	87	4.2e−50	Unknown protein (Arabidopsis gene ID K9I9.19)
17	TC128715	96	1.4e−52	Putative Pro-rich protein
18	TC115944	98	3.3e−96	Basic 30-kD endochitinase
19	TC116006	96	4.2e−145	Extensin
20	TC115911	99	2.9e−137	PR protein; P1(P14) protein; PR protein P6
21	TC115911	98	1.4e−166	PR protein; P1(P14) protein; PR protein P6
22	TC115911	93	5.5e−117	PR protein; P1(P14) protein; PR protein P6
23	TC124178	82	8.5e−74	Invertase-like protein
24	TC120032	95	5.4e−46	Unknown (Arabidopsis gene ID T27G7)
25	TC124672	57	2.1	TOM (target of myb1)-like protein
26	TC116590	99	1.8e−79	60S ribosomal protein L6
27	TC115998	89	1.1e−85	NP24 protein precursor; PR protein osmotin
28	TC115879	80	2.5e−42	60S ribosomal protein L41
29	TC115816	99	3.8e−96	Chitinase
30	TC115911	95	1.6e−110	PR protein; P1(P14) protein; PR protein P6
31	AW036273	72	9.0e−32	ID not assigned
32	AW094131	100	2.0e−46	Purple acid phosphatase-like protein
33	TC115944	90	3.5e−31	Basic 30-kD endochitinase
34	TC116997	88	8.4e−12	Ubiquitin-conjugating enzyme UBC2
35	TC124012	91	3.8e−85	TSI-1 protein
36	TC116821	86	1.4e−50	Acidic ribosomal protein P2b
37	TC115816	88	2.0e−66	Chitinase
38	TC115816	82	9.1e−57	Chitinase
39	TC115911	92	3.3e−97	PR protein; P1(P14) protein; PR protein P6
40	TC115816	98	1.6e−86	Chitinase
41	TC115787	88	4.0e−94	2-Oxoglutarate-dependent dioxygenase
42	TC116105	84	3.1e−81	Metallothionein-like protein
43	TC126387	98	1.5e−90	Proteinase inhibitor I
44	TC115911	93	2.3e−121	PR protein; P1(P14) protein; PR protein P6
45	TC115816	94	2.5e−76	Chitinase
46	TC115944	67	1.9e−42	Basic 30-kD endochitinase
47	TC115816	92	5.5e−86	Chitinase
48	TC115911	88	1.0e−96	PR protein; P1(P14) protein; PR protein P6

vitro (Van Hengel et al., 1998). Cell-surface AGPs may bind to linked ferulic acids in the cell wall to prevent the formation of diferulic acids, thereby allowing wall extensibility (Zhu et al., 1993). If the AGPs were degraded by chitinase, then enhanced peroxidase activity (see below) and diferulic acid cross-linking could contribute to stronger cell walls in *Cnr*.

In *Cnr* fruits, there was intense peroxidase activity not only in the skin and radial vascular network, but also within the parenchyma cells of the pericarp, columella, and fruit septa, and preliminary evidence suggests that a proportion of this activity is wall bound. Peroxidase is one of the enzymes most frequently implicated in catalyzing changes in the mechanical properties of plant cell walls (Fry, 1986; Lagrimini et al., 1993; Andrews et al., 2002). Recently,

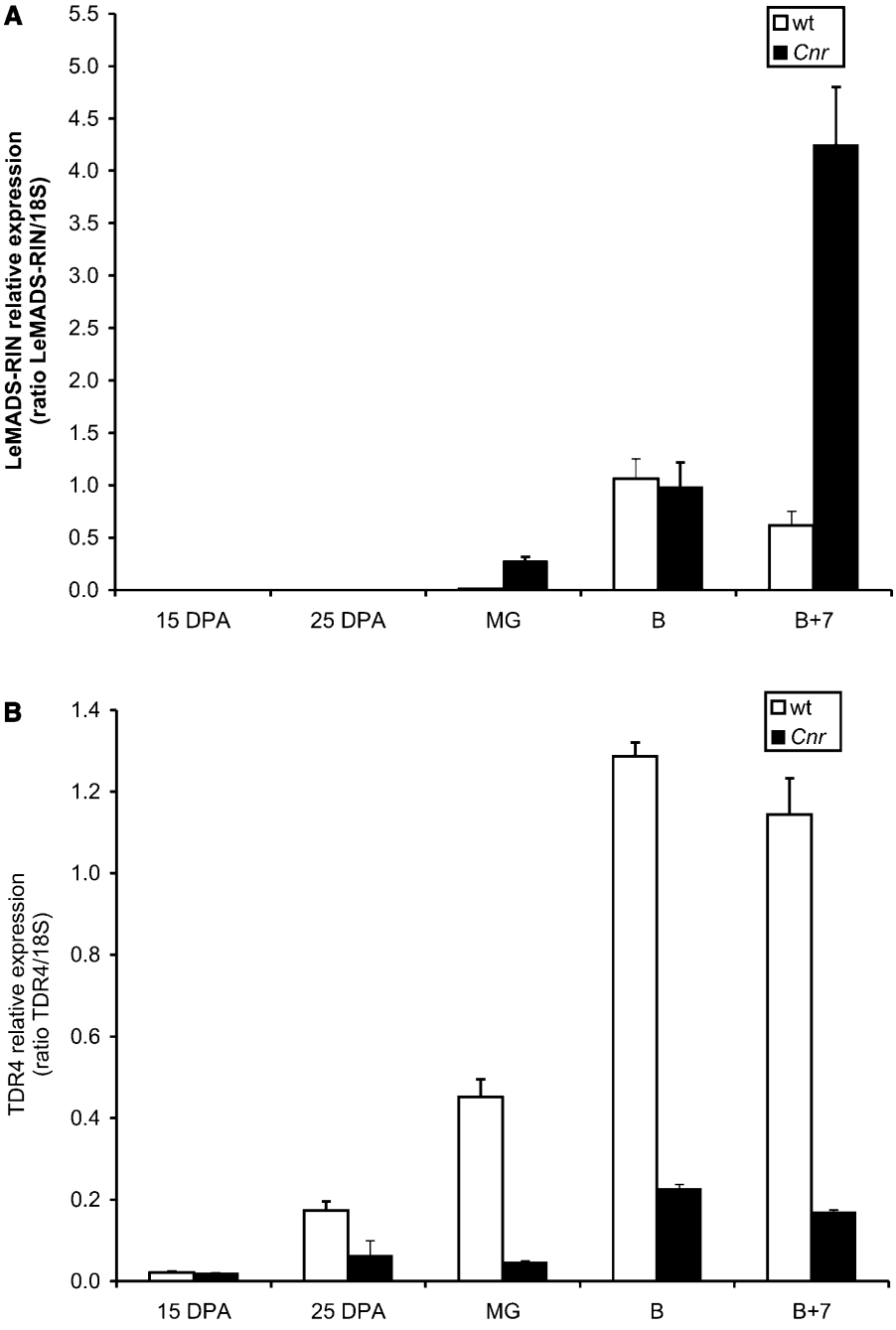

Figure 7. Real-time PCR determination of expression of (A) *Le MADS-RIN* (accession no. AF448523) and (B) *TDR4* (accession no. AY098732) during the development and ripening of wild-type and *Cnr* fruits.

it has been shown that cell wall-bound peroxidase isozymes are capable of changing the mechanical properties of the fruit tissue, possibly by catalyzing cross-linking and stiffening of cell walls (Andrews et al., 2002). The presence of these isozymes in the pericarp of *Cnr* may cause changes to the mechanical properties of parenchyma cell walls and contribute to the textural changes reported in *Cnr* (Orfila et al., 2001).

The microarray data provide additional evidence that the *Cnr* mutation severely affects ripening-related gene expression and, furthermore, has an effect on gene expression throughout fruit development. This includes premature induction of ripening-related gene expression in immature fruits. The array data must be treated with some caution since this preliminary experiment is based on pooled samples of RNA from several fruits and a single dual hybridization experiment. Further biological replication is required before a thorough statistical analysis can be undertaken and detailed interpretation of the results is valid. These initial results are, however, entirely consistent with the other molecular and biochemical studies and indi-

cate that mature *Cnr* fruits produce proteins more commonly associated with stress and wound responses rather than with ripening.

The failure of *Cnr* fruit to ripen may be due to misexpression of ripening-related genes early in fruit development compromising the normal program of gene expression. However, it is also likely that the failure of *Cnr* fruit to ripen reflects the dramatic reduction in the expression of a wide range of genes normally associated with ripening, including not only cell wall-degrading enzymes but also phytoene synthase, a likely PPR repeat-containing protein, acid invertase, alcohol dehydrogenase, lipoxygenase, ACC oxidase, and a putative t-SNARE SED5 gene among many others. The absence of phytoene and other carotenoid intermediates explains the abolition of carotenoid biosynthesis in *Cnr* (Fraser et al., 2001), and lowered PPR expression in the mutant may be related to alterations in the plastid translation machinery (Williams and Barkan, 2003). The reductions in acid invertase, alcohol dehydrogenase, and lipoxygenase point to an inhibition of the processes associated with flavor and aroma development (Klann et al., 1996; Speirs et al., 1998; Griffiths et al., 1999). The global gene expression profile from the microarray is entirely consistent with visual inspection of the *Cnr* phenotype, where it is evident that many aspects of normal ripening are compromised. These observations and the premature induction of some ripening genes in immature fruits suggest a central role for the gene at the *Cnr* locus in this important developmental process.

The differential screen identified a number of genes that were preferentially expressed in the *Cnr* mutant and similar to genes expressed in *Sambucus nigra* leaflets during ethylene-promoted abscission (Coupe et al., 1995, 1997). Twenty Sambucus abscission-related clones were isolated from a differential screen, and sequencing revealed that the majority encoded PR proteins, including a PR-1 protein, class I chitinase, proteinase inhibitors, and a metallothionein-like protein, all of which were matched by clones from the *Cnr* library. The specific function of PR proteins in this process is unclear, although it has been proposed that they are either involved in the cell separation process itself or protect the plant from pathogenic attack (del Campillo and Lewis, 1992).

Our current work on cloning the gene at the *Cnr* locus is aimed at allowing us to place the gene product in a framework that will describe the molecular regulation of ripening and permit connections between the downstream events in the cell wall and the regulatory factors controlling ripening. There is strong evidence emerging that certain MADS box genes play an important role in the ripening of fleshy fruits. Recently, the *rin* gene (*LeMADS-RIN*), which is essential for normal ripening in tomato, has been cloned and shown to be a member of the MADS box family (Vrebalov et al., 2002). In this study, we found that levels of *LeMADS-RIN* transcript were the same or greatly elevated in the mutant in the later stages of ripening. Furthermore, this study demonstrates that in the *Cnr* mutation, there is a reduction in the expression of the *TDR4* MADS box gene that is highly expressed during normal ripening. The expression of *TDR4* is also reduced in the nonripening mutants *rin* and *nor* (J. Giovannoni, personal communication). We propose that *CNR*, *RIN*, *NOR*, and *TDR4* are all part of the same regulatory network that is involved in controlling ripening in fleshy fruits.

Changes in *TDR4* expression in *Cnr* may be an important clue revealing a generic mechanism controlling cell separation in plant tissues. Studies on the dry fruits of Arabidopsis (*Arabidopsis thaliana*) have revealed that MADS box transcription factors are involved in the process of cell separation in siliques. These include *SHATTERPROOF1*, *SHATTERPROOF2*, and *FRUITFULL* (*FUL*; Liljegren et al., 2000; Ferrandiz et al., 2000). The *TDR4* gene sequence shows strong homology with the *FUL* gene from Arabidopsis and may be an ortholog (Litt and Irish, 2003). In Arabidopsis this MADS box gene is involved in the control of valve cell differentiation (the cells corresponding to the tomato pericarp). In the loss of function *ful* mutants, valve cells adopt the fate of dehiscence zone cells, which are normally programmed to undergo cell separation when the fruit matures (Ferrandiz et al., 2000). Overexpression of *FUL* produces plants with indehiscent fruit that fail to disperse their seeds normally. The role of the orthologs of *FUL* and other related genes in cell separation in fleshy fruits, if any, remains to be tested. If *TDR4* is an ortholog of *FUL*, it could act in tomato to suppress the formation of dehiscence zones in the fruit pericarp during ripening. By contrast, in *Cnr*, *TDR4* expression is suppressed, and this is correlated with abnormal levels of cell separation throughout the pericarp tissue.

Reduction of *TDR4* gene expression to around 10% of normal levels using antisense RNA resulted in no obvious fruit phenotypes, although there was an indication of an increase in cell wall stiffness determined by mechanical measurements (A. Popovich, E.M. Eriksson, and G.B. Seymour, unpublished data). The increase in cell wall stiffness is at least consistent with a role for *TDR4* in modulating cell wall structure. However, this experiment may have been confounded by functional redundancy of MADS box genes as reported recently for similar genes, including *AG* and *SHP* (Pinyopich et al., 2003; also see Litt and Irish, 2003), and, therefore, down-regulation of a number of related genes may be needed to see a strong effect. The *DEFH28* gene from Antirrhinum, which is expressed in the capsule walls and is very closely related to *TDR4* by sequence homology, can substitute for *FUL* in Arabidopsis (Müller et al., 2001). It would therefore be interesting (1) to determine whether *TDR4* can substitute for *FUL* in Arabidopsis and (2) to attempt to recover aspects of normal fruit texture in the *Cnr* background by up-regulating this gene in the mutant, and these experiments are in progress. That dry fruits such as siliques and fleshy fruits such as tomato could

share common mechanisms controlling aspects of cell separation is consistent with our understanding of the evolutionary relationship between fruit-bearing species. Fleshy fruits are likely to have evolved independently many times during the evolution of the angiosperms, but they are almost certainly derived from dry fruit types (Knapp, 2002). Utilizing information from Arabidopsis and tomato genomics resources may allow us to develop a model to predict approaches to controlling various aspects of ripening in a wide range of fleshy fruit-bearing species.

MATERIALS AND METHODS

Wild-type tomato fruits (*Lycopersicon esculentum* cv Ailsa Craig) and a near-isogenic line carrying the *Cnr* mutation (Orfila et al., 2001) were grown in a heated glasshouse using standard cultural practices with regular additions of N, P, K fertilizer and supplementary lighting when required. Plants were grown to three trusses. Flowers were tagged at anthesis, and fruits were harvested at the following stages: 15 DPA, 20 DPA, 25 DPA, MG (35–40 DPA), B, B + 3, and red ripe at B + 7. Fruits of the *Cnr* mutant were harvested at equivalent stages as determined by number of DPA.

Real-Time PCR Quantification

For quantitative PCR, total RNA was isolated from frozen fruit pericarp as described by Carey et al. (1995), and contaminating DNA was removed using DNA-free (Ambion, Huntingdon, UK) according to the manufacturer's instructions. First-strand cDNA was synthesized with the Omniscript RT kit (Qiagen, Crawley, UK), using equal amounts of oligo(dT) and random primers, according to the manufacturer's instructions. Real-time experiments were conducted in an iCycler iQ detection system (Bio-Rad, Hercules, CA), using the intercalation dye SYBRGreen I as a fluorescent reporter. Quantification of PCR products was performed via a calibration curve procedure using 18S RNA as an internal standard. PCR products were analyzed using melt curves as well as agarose gel electrophoresis, followed by gel extraction and DNA sequencing to ensure single product amplification. The ratio of gene-specific expression to 18S signal was defined as relative expression. PCR controls were performed in the absence of added reverse transcriptase to ensure RNA samples were free of DNA contamination.

Extraction of Cell Wall-Degrading Enzymes and Determination of Activity

Three individual fruits at each stage of ripeness were sampled and the assays performed in triplicate. Total cell wall-bound and soluble proteins were extracted from tomato pericarp with 1 M NaCl and precipitated with ammonium sulfate as described by Pressey (1983). Crude extracts from developing and ripening fruits were used for the estimation of PG, β-gal, and XET activity as described previously by Tucker et al. (1980), Carey et al. (1995), and Fry et al. (1992), respectively. PE activity was assayed in 0.5% citrus pectin (63%–66% esterified) and 5 mM Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl. Deesterification of the substrate was monitored by measuring the amounts of 0.1 M NaOH required to maintain pH 8.0 over 3 to 5 min. Activity was defined as mequivalents per minute. PE isoforms were separated by heparin affinity chromatography, using an Econo system (Bio-Rad, Hemel Hempstead, UK). The heparin columns (Bio-Rad) were prepared by washing with 5 mM Tris, pH 7.5, 300 mM NaCl at a rate of 1.7 mL/min, followed by 5 mM Tris, pH 7.5, 10 mM NaCl. PE isoforms were separated into 75 fractions by a salt gradient at a flow rate of 1 mL/min. Twenty microliters of each fraction was assayed for activity using a pH indicator in buffer (2 mM Tris-HCl, 0.15 M NaCl, 0.5% citrus pectin, 0.002% phenol red). Absorbance was measured at 405 nm, and profiles were created from endpoint readings between 0 and 3 h. Total protein was estimated by the dye-binding method of Bradford (1976), using bovine serum albumin as a standard.

Western-Blot Analysis

Western-blot analysis of crude cell wall and soluble proteins was performed by the method of Towbin et al. (1979), using a nitrocellulose membrane and a polyclonal antibody raised against purified PE2 protein (Hall et al., 1993).

Protein Sequencing

Crude protein extracts from *Cnr* B + 7 fruits were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membrane (Bio-Rad). Prior to transfer, the membrane was soaked briefly in methanol and then equilibrated in transfer buffer (10 mM CAPS, pH 11.0, 10% methanol) for 10 min. After transfer was complete, the blot was stained with ProBlot Coomassie Blue (0.1% Coomassie Blue R-250, 40% methanol, 1% acetic acid) and destained in 50% methanol. The bands of interest were excised from the dried blot and stored at −70°C prior to sequencing. The N-terminal amino acid sequence of the proteins was determined by automated Edman degradation chemistry, using the Procise 492 protein sequencer (Applied Biosystems, Warrington, Cheshire, UK).

Peroxidase Localization by Nitrocellulose Tissue Printing

Washed mature (B) fruits of wild type and *Cnr* were halved horizontally through the equatorial region of the fruits. The cut surfaces were rinsed repeatedly in 10 mM sodium acetate/citric acid buffer, pH 6.0, to reduce contamination of soluble (symplastic) material containing soluble peroxidase activity (Thompson et al., 1998). The cut surfaces were then lightly blotted onto filter paper before they were weighted down lightly onto nitrocellulose paper for 3 min to facilitate the binding of wall-bound protein. The tissue was then removed and the nitrocellulose paper stained for peroxidase activity using staining solution consisting of 100 mM sodium acetate/citric acid buffer, pH 6.0, containing 0.06% H_2O_2 and 0.06 mg mL^{-1} of chloronaphthol (freshly prepared at 0.3 mg mL^{-1} in methanol). The optimum results were achieved if the nitrocellulose paper was first presoaked in buffer containing 1 M NaCl for 15 min.

Preparation of a Tomato cDNA Microarray

A cDNA library was constructed from mRNA isolated from transgenic LC/C1 FM6203 tomato fruits (Bovy et al., 2002) harvested at B, turning, pink, and red stages. The library was made using the SMART cDNA library construction kit in the lambda Triplex vector (BD Biosciences/CLONTECH, Alphen aan den Rijn, The Netherlands), according to the manufacturer's protocol.

Recombinant clones were excised from the lambda phage in the phagemid vector pTRIPLEX2 by mass excision in the *Escherichia coli* SOL-R strain. A total of 2,018 randomly picked cDNA clones were sequenced from the 5′ end using the primer 5′-GGAAGCGCGCCATTGTGT and subsequently screened for homology with sequences present in the public databases by a BLAST search. Based on the sequence information, a total of 1,746 cDNA clones were selected for spotting onto microarray slides. These cDNA clones represented 1,055 different sequence contigs. Plasmid DNA was isolated from *E. coli* cultures harboring the 1,746 cDNA clones using Qiaprep Turbo miniprep kits (Qiagen). The plasmid inserts were amplified using two universal primers (5′-GGAAGCGCGCCATTGTGT and GCCAAGTGAGCTCGAATTGC), and the amplification products were checked on agarose gels for specificity and yield. The excess primers and free oligonucleotides were removed by column chromatography (Qiaquick PCR purification kits; Qiagen) using 100 μL of 1 mM Tris, pH 8.0, as elution buffer. Eluates were dried in a flow cabinet and dissolved in 10 μL of 5× SSC, giving a final DNA concentration of 0.5 to 1.0 $\mu g\ \mu L^{-1}$.

In addition, PCR fragments derived from the following sources were included on the microarray—(1) three non-plant genes as negative controls, used for the estimation of background signal: yeast Asp kinase (GenBank accession no. J03526), imidazoleglycerolphosphate dehydratase (accession no. Z75110), phosphoribo-sylaminoimidazole carboxylase (Z75036) (represented 12 times); (2) the complete coding sequence of the firefly luciferase gene (represented 4 times), and three partial luciferase clones encompassing the 5′, middle, and 3′ parts of the gene (each represented 4 times). As the samples were spiked with luciferase mRNA prior to labeling, this allowed for correction of the expression ratios for channel-specific effects. The partial

luciferase clones were additionally used to monitor the integrity of the labeled sample cDNA.

Microarrays were spotted on GAPS amino silane coated glass slides (Corning, Corning, NY) using a PixSys 7500 arrayer (Cartesian Technologies, Irvine, CA) equipped with Chipmaker 3 quill pins (Telechem, Sunnyvale, CA). Spotting volumes were about 0.5 nL, resulting in a spot diameter of 120 μm with a pitch of 160 μm. Each clone was spotted in duplicate 2.25 mm apart, resulting in a total spotted area of 9 $\times$ 9 mm. After spotting, the slides were rehydrated by holding them over a hot water bath (approximately 70°C), snap-dried on a 95°C to 100°C hot plate (5–10 s), and the DNA cross-linked using a UV cross-linker (150 mJ). The slides were soaked twice in 0.2% SDS for 2 min, twice in MilliQ water for 2 min, and transferred into boiling MQ water for 2 min to allow DNA denaturation. After thorough drying (5 min), the slides were rinsed 3 times in 0.2% SDS for 1 min, once in water for 1 min, submerged in boiling MQ water (2 s), and dried.

Microarray Hybridization Strategy

To compare the gene expression profiles in wild-type and *Cnr* fruits, RNA samples were prepared from pericarp of wild-type or *Cnr* fruits harvested at 15 DPA and B + 7, respectively. In addition, a common reference RNA sample was made by mixing aliquots of up to 20 poly(A^+) RNA samples, prepared from various tissues (leaves and fruit at different ripening stages) of untransformed and LC/C1 transgenic FM6203 tomato plants (Bovy et al., 2002). The four test RNA samples were reverse transcribed and labeled with Cyanine 3 (Cy3). Similarly, a bulk of reverse-transcribed reference RNA was labeled with Cyanine 5 (Cy5). Each labeled test cDNA sample was mixed with an aliquot of the common reference sample and analyzed in a dual hybridization experiment. The use of the same common reference sample in each hybridization allowed a direct comparison of all test samples with each other. A detailed description of the experimental procedures is explained below.

Preparation of RNA Samples, Labeling, and Microarray Hybridization

RNA samples were prepared from pericarp tissue pooled from at least three fruits. Total RNA was isolated from frozen tissue, as described by Carey et al. (1995). mRNA was purified using Dynabeads Oligo$(dT)_{25}$ (Dynal A.S, Oslo) according to the manufacturer's protocol. Aliquots of 2.5 μg of poly(A^+) RNA were spiked with 1.0 ng of in vitro synthesized luciferase mRNA (Promega, Madison, WI) and reverse transcribed in the presence of 5-(3-aminoallyl)-2′-dUTP (Sigma A 0410; Sigma, St. Louis) using 2 μg of oligo$(dT)_{21}$ as a primer. A 25-μL reaction containing, in addition to the oligo(dT)-annealed RNA template, 1 μL of first-strand buffer (Life Technologies, Paisley, Scotland), 10 mM dithiothreitol, 15 units of ribonuclease inhibitor (Life Technologies), 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.3 mM dTTP, 0.2 mM aminoallyl-dUTP, and 150 units of SuperScript II RNase H-reverse transcriptase (Life Technologies) was incubated at 37°C for 2 h. Nucleic acids were then ethanol precipitated at room temperature and dissolved in 10 μL of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Next, cDNA/mRNA hybrids were denatured (3 min at 98°C) and chilled on ice. RNA was degraded by adding 2.5 μL of 1 M NaOH and incubating 10 min at 37°C. After adding 2.5 μL of 1 M HEPES, pH 6.8, and 2.0 μL of 1 M HCl, the cDNA was recovered by ethanol precipitation and resuspended in 10 μL of 0.1 M sodium carbonate buffer, pH 9.3.

In a second step, the modified cDNA was coupled to a fluorescent dye, either Cy3 or Cy5, using reactive Cy3- or Cy5-NHS-esters (Amersham Pharmacia, Little Chalfont, Bucks, UK). To this end, 10 μL of 10 mM dye (in dimethyl sulfoxide) was added to 10 μL of the cDNA sample and incubated at room temperature for 30 min. Finally, the labeled cDNA was ethanol precipitated twice and dissolved in 5 μL of water. Following prehybridization at 42°C for 2 h in a few mL of hybridization buffer (50% formamide, 5$\times$ Denhardt's reagent, 5$\times$ SSC, 0.2% SDS, 0.1 mg mL^{-1} denatured fish DNA), slides were rinsed in MQ water and in isopropanol and then dried by centrifugation (1 min, 470*g*). For a dual hybridization, 80 μL of hybridization mixture, containing both a Cy3- and a Cy5-labeled sample at a concentration corresponding to 8 ng (Cy3) or 2 ng (Cy5) of the initial mRNA per microliter of mixture, was used. Prior to use, the hybridization mixture was heated at 95°C (1 min), cooled on ice, and centrifuged to remove any debris. Hybridizations were performed overnight at 42°C using a GeneFrame (15 $\times$ 16 mm, 65-μL volume; ABgene AB-0577) in a hybridization chamber. After hybridization, slides were washed at room temperature in 1$\times$ SSC, 0.1% SDS (5 min) followed by 0.1$\times$ SSC, 0.1% SDS (5 min) and rinsed briefly in 0.1$\times$ SSC before drying by centrifugation (1 min, 470*g*).

Microarray Data Acquisition and Analysis

Slides were scanned using a ScanArray 3000 (Packard BioScience, Monza, Italy) at 75% laser power and 75% attenuation at a resolution of 10 μm. The resulting Cy3 and Cy5 images were stored as TIFF files. Total pixel intensities within a fixed area (circle, diameter 12 pixels) were obtained for each spot using ArrayVision image analysis software (Imaging Research, St. Catherines, Canada). Next, average background values, calculated from the hybridization signals of the non-plant clones, were subtracted to correct for nonspecific fluorescence. Signals not reaching 1.5$\times$ background were filtered out. Normalization of the two samples in each hybridization was done using the mean hybridization signal of the full-length luciferase clones, resulting from the spiked luciferase mRNA. Finally, the expression ratios, that is, the expression in the sample under study compared with the reference sample, were calculated for each clone. The reference sample was the same for every hybridization, allowing for direct comparison of all hybridization experiments. Expression ratios for the spotted duplicates (A, B) were calculated separately, and the average of both values was used for further analysis. Duplicates failing to meet the criteria (^{2}Log (A/B) < 1) were filtered out. Using a pairwise comparison of all four samples, clones were selected showing a 2-fold or more difference in at least one of the comparisons. Subsequently, redundant clones were filtered out based on sequence identity, BLAST homology, and expression pattern. Hierarchical clustering (Pearson algorithm) was done using GeneMaths software (version 2.0; Applied Maths, Sint-Martens-Latem, Belgium).

Differential Screen

mRNA was isolated from total *Cnr* RNA and used to construct a cDNA library in the ZAP Express vector and the library was packaged following the manufacturer's instructions (Stratagene, Amsterdam). Differential screening was performed with radiolabeled single-stranded cDNA probes from the wild-type or *Cnr* tissue. Plaques that differentially hybridized to the mutant cDNA probe were cored from the plates, and the cDNA inserts were amplified from denatured phage particles by PCR using the T3 forward and T7 reverse primers. The PCR products were sequenced by the Sanger dideoxynucleotide chain termination method (Sanger et al., 1977) and compared to the TIGR (www.tigr.org) and Solanaceae Genomics Network (www.sgn.cornell.edu) databases for homology, using the BLASTn algorithm (Altschul et al., 1997).

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