Reprogramming the Phenylpropanoid Metabolism in Seeds of Oilseed Rape by Suppressing the Orthologs of REDUCED EPIDERMAL FLUORESCENCE1

Juliane Mittasch, Christoph Böttcher, Andrej Frolov, Dieter Strack, and Carsten Milkowski*

Department of Secondary Metabolism (J.M., A.F., D.S.) and Department of Stress and Developmental Biology (C.B.), Leibniz Institute of Plant Biochemistry, D–06120 Halle (Saale), Germany; and Interdisciplinary Center for Crop Plant Research, Martin Luther University Halle-Wittenberg, D–06120 Halle (Saale), Germany (C.M.)

As a result of the phenylpropanoid pathway, many Brassicaceae produce considerable amounts of soluble hydroxycinnamate conjugates, mainly sinapate esters. From oilseed rape (Brassica napus), we cloned two orthologs of the Arabidopsis (Arabidopsis thaliana) gene REDUCED EPIDERMAL FLUORESCENCE1 (REF1) encoding a coniferaldehyde/sinapaldehyde dehydrogenase. The enzyme is involved in the formation of ferulate and sinapate from the corresponding aldehydes, thereby linking lignin and hydroxycinnamate biosynthesis as a potential branch-point enzyme. We used RNA interference to silence REF1 genes in seeds of oilseed rape. Nontargeted metabolite profiling showed that BnREF1-suppressing seeds produced a novel chemotype characterized by reduced levels of sinapate esters, the appearance of conjugated monolignols, dilignols, and trilignols, altered accumulation patterns of kaempferol glycosides, and changes in minor conjugates of caffeate, ferulate, and 5-hydroxyferulate. BnREF1 suppression affected the level of minor sinapate conjugates more severely than that of the major component sinapine. Mapping of the changed metabolites onto the phenylpropanoid metabolic network revealed partial redirection of metabolic sequences as a major impact of BnREF1 suppression.

Phenylpropanoid metabolism provides plants with a vast array of phenolic compounds that contribute to nearly all aspects of plant life (Vogt, 2010). In species of the Brassicaceae family, soluble hydroxycinnamate conjugates, mainly sinapate esters, constitute an abundant metabolite fraction produced from a branch of the phenylpropanoid pathway (Fraser and Chapple, 2011). As major compounds, sinapoylmalate accumulates in leaves (Hause et al., 2002) and sinapoylcholine (sinapine) in seeds (Bouchereau et al., 1991; Fig. 1).

In Arabidopsis (Arabidopsis thaliana), the disturbed accumulation of sinapoylmalate caused the mutant phenotype reduced epidermal fluorescence (ref; Ruegge and Chapple, 2001). Molecular characterization of the ref1 mutant led to the identification of the gene At3g24503 (REF1) encoding coniferaldehyde dehydrogenase/sinapaldehyde dehydrogenase (CALDH/SALDH; EC 1.2.1.68; Nair et al., 2004). The bifunctional enzyme CALDH/SALDH was shown to catalyze the NADP+-dependent oxidation of coniferaldehyde and sinapaldehyde to yield the corresponding hydroxycinnamates ferulate and sinapate. As a potential branching enzyme, the enzymatic activity of the REF1-encoded CALDH/SALDH might be crucial for the partition ratio of metabolites between lignin and hydroxycinnamate biosynthesis (Fig. 1). Therefore, manipulation of REF1 expression coupled to a comprehensive metabolite analysis of transgenic plants appeared as an interesting strategy to gain a deeper understanding of the plant phenylpropanoid metabolic network. Moreover, in crop plants like oilseed rape (Brassica napus), where high levels of sinapate esters contribute to antinutritive features, the suppression of REF1 orthologs might cause increased quality.

This work describes the isolation of REF1 orthologous genes from oilseed rape (BnREF1). By RNA interference, transgenic lines of oilseed rape were generated that suppress BnREF1 gene expression during seed development. Homozygous transgenic progeny were developed and used in a comprehensive liquid chromatography-mass spectrometry (LC/MS)-based metabolite profiling to investigate the impact of BnREF1 silencing in seeds. Mapping of changed metabolite patterns onto the phenylpropanoid metabolic network revealed insight into the molecular mechanisms by which silencing of BnREF1 produced a novel seed chemotype in oilseed rape.
homologs were produced by RACE-PCR with poly(A+) levels in roots, the corresponding full-length cDNA indicated for the Arabidopsis gene high expression gator (http://www.genevestigator.com/gv/plant.jsp) gov/unigene). Since database analyses by Genevestigator (http://www.ncbi.nlm.nih.gov/unicluster) and direct partial DNA digestion or the occurrence of \( BnREF1 \)-related sequence motifs within the complex \( B. rapa \) spp. genomes than in Arabidopsis.

Comparison of the coding sequences showed that \( BnREF1\_\text{II} \) was distinguished by only two nucleotide substitutions from \( Bra001952 \), whereas \( BnREF1\_\text{I} \) displayed 25 substitutions. This indicates that \( BnREF1\_\text{II} \) represents the \( B. rapa \) A genome homolog within the allotetraploid oilseed rape genome (genome organization: AACC, \( 2n = 38 \); U, 1935).

To estimate the number of \( BnREF1 \) gene loci in oilseed rape, a genomic Southern-blot analysis was performed. Therefore, genomic DNA of oilseed rape and its diploid progenitors turnip (AA, \( 2n = 20 \)) and kale (\( B. oleracea \); CC, \( 2n = 18 \)) was digested with restriction endonuclease EcoRI or HinIII and hybridized with a 419-bp probe amplified from \( BnREF1\_\text{I} \) cDNA. The probe covered sequences of exon 2 and exon 3, which were not cleaved by EcoRI or HinIII (Fig. 2). The high sequence identity with \( BnREF1\_\text{II} \) of 98% in the covered region ensured that the probe recognizes both \( BnREF1\_\text{I} \) and \( BnREF1\_\text{II} \). EcoRI-digested genomic DNAs gave rise to two hybridized bands in oilseed rape, which corresponded in size with the signal produced either from turnip or kale (Fig. 3). In addition, we detected some larger bands, which might reflect partial DNA digestion or the occurrence of \( BnREF1\)-related sequence motifs within the complex \( B. rapa \) spp. genomes. Hybridization of HinIII-digested genomic DNAs produced two signals from oilseed rape. The larger hybridization band from oilseed rape corresponded in size with the single band produced from kale. For the smaller hybridization band from oilseed rape, we calculated a size of 1.754 kb, as indicated from the genomic sequence of \( BnREF1\_\text{II} \). In comparison, the HinIII-digested DNA from turnip produced a larger faint hybridization band in the size range calculated from the sequence of \( Bra001952 \) (2.487

**RESULTS**

**The Genome of Oilseed Rape Harbors Two**

**BnREF1 Homologs**

Reverse transcription (RT)-PCR with degenerated primers derived from Arabidopsis \( REF1 \) (\( AtREF1\_\text{I}; At3g24503 \)) was used to isolate a 590-bp partial complementary DNA (cDNA) from seeds of oilseed rape. Sequence analysis revealed the isolated cDNA fragment as a member of Unigene cluster \( Bna.2995 \) that is sequence identity to \( AtREF1 \) (http://www.genevestigator.com/gv/plant.jsp).

Since database analyses by Genevestigator (http://www.genevestigator.com/gv/plant.jsp) indicated for the Arabidopsis gene high expression levels in roots, the corresponding full-length cDNA homologs were produced by RACE-PCR with poly(A+) RNA from roots of young oilseed rape plants. The generated amplicons represented two highly similar but distinct sequence variants designated as \( BnREF1\_\text{I} \) and \( BnREF1\_\text{II} \). The cDNAs of \( BnREF1\_\text{I} \) and \( BnREF1\_\text{II} \) shared 98% sequence identity. \( BnREF1\_\text{I} \) carried the complete sequence of the initially isolated 590-bp EST from seeds of oilseed rape. An electronic northern-blot analysis confirmed the expression of both \( BnREF1\_\text{I} \) and \( BnREF1\_\text{II} \) in seeds of oilseed rape (Supplemental Fig. S1). Of the 100 \( BnREF1 \) ESTs provided by public databases, 60% originated from seeds, whereas seedlings, roots, leaves, and anthers together accounted for the residual 40%. Within the fraction of seed ESTs, sequences of \( BnREF1\_\text{I} \) and \( BnREF1\_\text{II} \) were present with almost similar frequency.

PCR was used to amplify the genomic sequences of \( BnREF1\_\text{I} \) and \( BnREF1\_\text{II} \). Sequence comparison with \( AtREF1 \) and \( Bra001952 \), a \( REF1 \) ortholog found in the recently published genome sequence of turnip (\( B. rapa \); http://www.brassicadb.org/brad/; Wang et al., 2011), revealed similar exon-intron patterns (Fig. 2). Striking differences were found for introns 1 and 3, which covered significantly more nucleotides in the \( B. rapa \) spp. genes than in Arabidopsis.

Figure 1. Biosynthesis of sinapate esters emphasizing the crucial role of the bifunctional hydroxycinnamaldehyde dehydrogenase CALDH/SALDH encoded by the gene \( REF1 \). Dashed arrows symbolize multistep biosyntheses. Abbreviations for enzymes are as follows: CAD, (hydroxy)cinnamyl alcohol dehydrogenase; SAD, sinapyl alcohol dehydrogenase; SCT, 1-O-sinapoylglucosyltransferase; SGT, UDP-Glc: sinapate glucosyltransferase; SMT, 1-O-sinapoylglucosyl:malate sinapoyltransferase.
kb). Sequence comparison revealed this signal shift as a consequence of the expanded intron 1 in \textit{Bra001952}, which was accompanied by the loss of a \textit{Hin}dIII restriction site (Fig. 2). Therefore, the hybridization signals, which appear at 1.754 kb in oilseed rape and in the range of 2.5 kb in turnip, can be considered as corresponding bands. As a result, the genomic Southern-blot analyses confirmed the presence of two genomic \textit{BnREF1} loci in oilseed rape harboring the genes \textit{BnREF1}_I and \textit{BnREF1}_II. The genomes of turnip and kale each contain a single \textit{REF1} locus.

\textbf{\textit{BnREF1} Encodes a Bifunctional CALDH/SALDH}

The coding sequence of \textit{BnREF1}_I was cloned into the bacterial expression plasmid pQE30 and used to transform \textit{Escherichia coli}. Crude protein extracts of \textit{E. coli} expressing \textit{BnREF1}_I were assayed for dehydrogenase activity toward coniferaldehyde and sinapaldehyde. HPLC analysis of assay mixtures revealed the production of ferulate from coniferaldehyde and of sinapate from sinapaldehyde (Fig. 4). The enzymatic activity assessed from product formation in crude protein extracts indicated that \textit{BnREF1}_I encodes a bifunctional hydroxycinnamaldehyde dehydrogenase with catalytic activities toward coniferaldehyde and sinapaldehyde.

\textbf{Suppression of \textit{BnREF1} Restrained Sinapate Ester Accumulation in Seeds}

For seed-specific down-regulation of \textit{BnREF1} expression, a partial cDNA sequence from \textit{BnREF1}_I was used to assemble a hairpin cassette fused to the seed-specific napin promoter (Kridl et al., 1991). The resulting plasmid construct \textit{pLH-REF1i} designed to silence both \textit{BnREF1}_I and \textit{BnREF1}_II genes via seed-specific double-stranded RNA interference was transformed into oilseed rape ‘Lisora’. Twenty-four phosphinothricine (PPT)-resistant transgenic lines (T1 generation) were subjected to further analysis. To estimate the number of transgenic \textit{BnREF1i} loci, genomic Southern-blot analyses of T1 plants were carried out.

All T1 plants were selfed to produce T2 seeds. In a single seed analysis, 10 individual seeds of each of the segregating T2 progeny were subjected to ultra-performance liquid chromatography (UPLC)-based quantification of sinapine and of total sinapate content (Fig. 5). Median values of sinapine and total sinapate contents per individual T2 progeny clearly demonstrated the suppression effect produced by the \textit{BnREF1i} approach. Transgenic lines harboring the \textit{BnREF1i} insertion (\textit{BnREF1i} lines) displayed decreased median values for both the sinapine (Fig. 5A) and the total sinapate (Fig. 5B) contents compared with the non-transformed lines. Among the transgenic lines, multi-copy insertion lines showed a trend toward low median values, whereas single-copy insertion lines preferentially fell into the group of intermediate median values. This was in accordance with the expected ratio of individual \textit{BnREF1i} seeds in the segregating T2 progeny. The minimum values of this single-seed analysis revealed the strength of suppression achievable in each line. Interestingly, in all transgenic seeds, the decrease was more pronounced in the fraction of total sinapate esters than in the sinapine content alone.

Since downstream metabolic investigation by comprehensive LC/MS depends on stable transgenic lines, further analyses relied on single-copy insertion lines, which could be propagated to homozygosity. Accordingly, we selected the transgenic population for single-

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{Gene structures of oilseed rape genes \textit{BnREF1}_I and \textit{BnREF1}_II compared with the Arabidopsis homolog \textit{AtREF1} and the \textit{REF1} gene model \textit{Bra001952} retrieved from the genome data of turnip (http://brassicadb.org/brad/). \textit{BnREF1}_I and \textit{BnREF1}_II were isolated from oilseed rape ‘Lisora’. Gene model \textit{Bra001952} is based on sequence data of turnip ‘Chiifu-401’. Exons are depicted by boxes. Introns 1 and 3 differing markedly in size between \textit{Brassica} spp. and Arabidopsis are highlighted. The sizes of these introns are given as numbers of nucleotides. Genes are drawn to scale. Black bars indicate the sequence regions in \textit{BnREF1}_I and \textit{BnREF1}_II recognized by the cDNA probe in genomic Southern hybridization (Fig. 3). E, \textit{EcoRI} recognition site; H, \textit{HindIII} recognition site.}
\end{figure}
plants was cleaved by restriction endonucleases EcoRI and HindIII. After separation by agarose gel electrophoresis and immobilization onto a nylon membrane, the DNA fragment patterns were hybridized with a DIG-labeled PCR-derived DNA probe covering a partial sequence of BnREF1 including parts of exons 2 and 3 (Fig. 2). For the restriction endonucleases EcoRI and HindIII, the probe sequence did not carry recognition sites. The arrows point to the bands of oilseed rape at similar marker positions where their orthologs in kale (dashed arrows) and turnip (solid arrows) were located. For DIG-labeled DNA molecular weight markers (M), the fragment sizes are given in bp.

Figure 3. Southern-blot detection of REF1 genes in oilseed rape (B. napus), kale (B. oleracea), and turnip (B. rapa). Genomic DNA of the plants was isolated by restriction endonucleases EcoRI and HindIII. After separation by agarose gel electrophoresis and immobilization onto a nylon membrane, the DNA fragment patterns were hybridized with a DIG-labeled PCR-derived DNA probe covering a partial sequence of BnREF1 including parts of exons 2 and 3 (Fig. 2). For the restriction endonucleases EcoRI and HindIII, the probe sequence did not carry recognition sites. The arrows point to the bands of oilseed rape at similar marker positions where their orthologs in kale (dashed arrows) and turnip (solid arrows) were located. For DIG-labeled DNA molecular weight markers (M), the fragment sizes are given in bp.

Copy insertion lines that displayed a clear suppression phenotype in T2 seeds, as indicated by intermediate median values and lowest single-seed values for both the sinapine and sinapate ester contents. Considering these criteria, the results revealed transgenic lines 29b and 27a as the only candidates for downstream analyses (Fig. 5). Individual T2 seeds of line 29b displayed a reduction of the sinapine content to about 56% (1.27 mg g⁻¹ seed) and of sinapate esters to about 33% (0.94 mg g⁻¹ seed) of the lowest values observed in nontransformed lines (sinapine content in line 27b, 2.25 mg g⁻¹ seed; sinapate ester content in line 5, 2.84 mg g⁻¹ seed). Transgenic line 27a produced individual T2 seeds with reduced contents of sinapine and sinapate esters to about 57% and 45% of the lowest control values.

The presence of single-copy transgenic loci in lines 27a and 29b was confirmed by segregation analyses of the T2 progeny. From both lines, a set of individual T2 seeds were germinated. At 10 d after germination, one cotyledon was harvested from each seedling and used for the isolation of genomic DNA. Genotyping was done by PCR amplification of a 658-bp DNA covering the antisense part of the silencing cassette at the transfer DNA part of pLH-REF1i (Fig. 6, A and B). Out of the 20 T2 individuals tested for line 27a, 15 carried the expected genomic insertion. For line 29b, 18 out of 23 genotyped T2 individuals produced the appropriate PCR signal. After the emergence of primary leaves at 14 d after germination, the T2 seedlings were treated with PPT to prove segregation of the transgene-mediated resistance. In both transgenic lines, the PPT resistance was directly linked to the genomic BnREF1i insertion (i.e. the individuals carrying BnREF1i developed the resistance phenotype; Fig. 6, B and C). BnREF1i insertion and PPT resistance segregated with a statistically significant ratio of 3:1 (insertion positive and resistant:without insertion and sensitive). This confirmed the presence of single-copy BnREF1i loci in both transgenic lines 27a and 29b. Consequently, PPT-resistant T2 individuals of lines 27a and 29b were propagated and selfed for the production of homozygous T3 progeny.

To prove the impact of seed-specific BnREF1i suppression in the first homozygous generation, T3 seeds of lines 27a and 29b were subjected to HPLC-based quantification of the sinapate ester content (Fig. 7A). Seeds of line 29b displayed significantly decreased amounts of accumulating sinapine and sinapate esters. The sinapine content in this line was reduced to about 55% and the sinapate ester content to about 35% of the nontransformed wild type. Surprisingly, our analyses indicated that the T3 seeds of line 27a had recovered sinapine and sinapate ester contents in the range of the wild type. Likewise, the CALDH/SALDH enzymatic activities were down-regulated only in line 29b (Fig. 7B). The CALDH/SALDH activities in seeds of line 29b were about 67% and 41% of the wild type. However, in seeds of line 27a, the CALDH/SALDH enzymatic activities were in the range of the wild-type levels. To elucidate the molecular reason for the relieved suppression in line 27a, we analyzed the structure of the BnREF1i transgenic locus in the T3 progeny. A detailed PCR-based analysis revealed a structural change within the integrated BnREF1i suppression cassette of line 27a (Fig. 7C). Our analyses detected that the sense arm of the original hairpin construct had been lost from the BnREF1i transgenic locus, thus resulting in an incomplete suppression construct in line 27a. The resulting incomplete suppression cassette of line 27a T3 progeny obviously no longer mediated BnREF1i silencing. This was in accordance with the expression of BnREF1i assessed by semiquantitative RT-PCR (Supplemental Fig. S2).

The Low-Sinapate-Ester Trait of BnREF1i Seeds Was Associated with a Changed Phenylpropanoid Pathway

T4 seeds of the transgenic lines 27a and 29b and the corresponding wild-type oilseed rape ‘Lisora’ were...
subjected to nontargeted LC/MS-based metabolite profiling. To enable a comprehensive analysis of semipolar seed metabolites, a previously developed two-step extraction/fractionation procedure was applied for sample preparation (Clauss et al., 2011). Low concentrated methanolic total extracts (2.5 mg fresh weight mL$^{-1}$) were prepared from which the abundant phenolic choline esters and related compounds were pro-ferred by ultra-performance liquid chromatography-coupled electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC/ESI-QTOFMS) in positive ion mode. For the analysis of compounds with lower abundance, choline ester-depleted extracts with a 10-fold increased concentration (25 mg fresh weight mL$^{-1}$) were employed. In these extracts, the detection of metabolites proved to be most efficient by the use of UPLC/ESI-QTOFMS in negative ion mode.

In order to gain a first insight into the acquired data sets, principal component analyses were performed. For both total and choline ester-depleted extracts, the first two principal components described 83% and 84% of total variance, respectively. The first principal component (79%–80% of total variance) revealed for both types of extracts a clear discrimination of transgenic line 29b from the wild type and transgenic line 27a (Fig. 8A). To identify metabolites accounting for this characteristic discrimination, pairwise comparisons between metabolite profiles of line 29b and the wild type as well as line 29b and line 27a were carried out and intersections were calculated (Fig. 8B). For total extracts, the intensities of 308 out of 1,814 detected molecular features (unique mass-to-charge retention time pairs) were shown to be significantly decreased in line 29b compared with both the wild type and line 27a. On the other hand, 240 molecular features were increased in line 29b relative to the wild type and line 27a. Analogously, 895 out of 4,564 molecular features detected in choline ester-depleted extracts had decreased intensities in line 29b after comparison with the other two lines, whereas 655 showed an increased intensity. Based on these differential molecular feature sets, compound mass spectra were reconstructed and used in combination with individually acquired collision-induced dissociation mass spectra for annotation of the underlying compounds. A total of 101 metabolites were annotated, of which 28 could be unambiguously identified by comparison with authentic standards (Supplemental Table S2; annotation level 1). For 58 metabolites, putative structures were deduced from elemental compositions and fragmentation patterns (annotation level 2). Seven metabolites could be assigned to a putative compound class (annotation level 3), whereas eight remained unknown (annotation level 4). Mass spectral data and relative quantification of all annotated metabolites are given in Supplemental Table S3 and Supplemental Figure S3, respectively. Molecular structures of the identified and putatively annotated metabolites are shown in Supplemental Figure S3. Among the 101 total annotated compounds, 51 could be classified as novel metabolites, which, to our knowledge, have not been reported for oilseed rape so far. The majority of these novel metabolites appeared in seeds of transgenic line 29b.

Supplemental Figure S4 shows a heat-map representation of relative quantification of the annotated metabolites in wild-type and transgenic lines 27a and 29b. The metabolic profile of wild-type seeds is characterized by a pronounced accumulation of sinapine.
(sinapoylcholine [compound 19]; Supplemental Table S2; Fig. 9), which is accompanied by significant amounts of 1-O-sinapoyl-β-glucose (62), 2-O-sinapoylmalate (54), 1,2-di-O-sinapoyl-β-glucose (66), and sinapoylated kaempferol glycosides (46–50). In agreement with the analysis of sinapine and saponifiable sinapate in T3 seeds, seeds of line 29b showed a striking decrease of the levels of all these major sinapate esters in relation to wild-type seeds. The sinapine level of line 29b was reduced to 42% of the wild-type level. As a corollary of its reduced formation, a moderate increase in the level of free choline (1) was detected. The amount of the metabolic precursor of sinapine and numerous other sinapate esters, 1-O-sinapoyl-β-glucose (62), was reduced to approximately 1% of the wild-type level. A similar low amount was observed for 2-O-sinapoylmalate (54; approximately 4% of the wild-type level) and a less decreased one for 1,2-di-O-sinapoyl-β-glucose (peak 66; approximately 24% of the wild-type level). Additionally, severely reduced levels of all sinapoylated kaempferol triglycosides and tetrarosides were observed for line 29b when compared with the wild type. In contrast, relevant amounts of kaempferol 3-O-sophoroside (peak 42) and its sinapyl conjugate (peak 43) were found to hyperaccumulate in seeds of line 29b, whereas the level of kaempferol 3-O-sophoroside-7-O-glucoside (peak 44) remained constant.

The applied metabolite profiling approach allowed a detailed analysis of minor compounds and conferred a comprehensive insight into the metabolic changes triggered by the suppression of BnREF1 (Fig. 10). Seeds of line 29b accumulated sinapyl alcohol-derived monolignol, dilignol, and trilignol conjugates (peaks 77–91). Fifteen such conjugates were detected and considered as novel metabolites in oilseed rape. Among them, 8,8-coupled homodimers (peaks 78–80), 4-O-8-coupled
homodimers (81–84), and a trimer (85) of sinapyl alcohol, as well as 4-O-8-coupled heterodimers of sinapyl alcohol and 7,8-dihydrosinapyl alcohol (87–91), were identified as sulfated or malonylated hexose conjugates. In addition, we found increased levels of 4-O-8 cross-coupling products of sinapine and sinapyl alcohol (23 and 25) and of feruloylcholine and sinapyl alcohol in line 29b (8 and 12). The suppression of BnREF1 also impacted ferulate conjugates, which typically accumulate at low levels in wild-type seeds. The amounts of 1-O-feruloyl-β-glucose (59) and 2-O-feruloylmalate (53) were strongly reduced in line 29b, as observed for the corresponding sinapate esters. The amount of feruloylcholine (5), however, was almost unaltered.

In contrast to the observed accumulation of sinapyl alcohol conjugates in seeds of line 29b, analysis of the differential molecular features gave no clear indication of an analogous accumulation of coniferyl alcohol conjugates. The levels of 4-O-8 cross-coupling products between sinapine and conifer alcohol were unaltered (22) or increased (24) in line 29b in comparison with the wild type, whereas the amounts of 4-O-8 and 5-8 cross-coupling products of feruloylcholine and coniferyl alcohol (7, 9, and 11) were entirely decreased. Interestingly, our analyses detected a significant decrease in the amounts of 1-O-5-hydroxyferuloyl-glucose (60) and 5-hydroxyferuloylcholine (18) in line 29b in relation to the wild type. Another interesting result of REF1 suppression in seeds of line 29b was the decreased level of the cyclic bis-isofuruloyl sperridine conjugate (39).

**DISCUSSION**

**Suppression of BnREF1 Interferes with Sinapate Ester Accumulation**

In seeds of oilseed rape, the suppression of BnREF1 was shown to affect more severely the accumulation of minor sinapate esters than that of sinapine (Figs. 7 and 9). This was surprising, since the suppression of BnREF1 primarily reduces the metabolic pool of sinapate, which feeds into both the synthesis of sinapine and of other sinapate esters (Fig. 1). Accordingly, the less pronounced inhibition of sinapine accumulation indicates that in seeds of oilseed rape, the formation of sinapine is the preferred pathway of sinapate ester metabolism. So far, the molecular mechanisms governing the preference of sinapine biosynthesis over competing syntheses remain elusive. Hypothetical mechanisms would include altered substrate availability by the existence of different subcellular sinapate pools for distinct pathways, differences in the substrate affinity or the cellular abundance of branching enzymes, suppression of competing pathways by sinapine, as well as metabolic channeling of sinapine biosynthesis. Previous work has produced evidence of metabolon formation in early phenylpropanoid biosynthesis leading to efficient metabolic channeling (Winkel-Shirley, 1999;
Metabolic Redirection Triggered by BnREF1 Suppression

Metabolic profiling of fractionated seed extracts, which had been depleted of the abundant choline esters, allowed a comprehensive analysis of phenylpropanoid-derived metabolites that accumulate to minor concentrations. Although quantitative changes in this metabolite fraction did not substantially affect the net decrease of sinapate moieties, they provide detailed insight into the metabolic changes triggered by the suppression of BnREF1 (Fig. 10). In seeds of line 29b, the inhibited accumulation of sinapate esters (19, 54, 62, and 66) was a result of the reduced capability to convert sinapaldehyde into sinapate (51). Instead, sinapaldehyde was increasingly converted to sinapyl alcohol, leading to the accumulation of sinapyl alcohol-derived monolignol, dilignol, and trilignol conjugates.
This supports the hypothesis of a partial redirection of the metabolic flux from the sinapate ester branch toward sinapyl alcohol upon BnREF1 suppression. In analogy, the decreased levels of feruloylated compounds (7, 9, 11, 18, 53, and 59) reflected the down-regulated synthesis of ferulate caused by REF1 suppression in oilseed rape line 29b. In contrast to the suppressed SALDH activity, the down-regulated CALDH activity did not lead to an accumulation of coniferyl alcohol-derived lignols in seeds of line 29b. A potential excess of coniferaldehyde or coniferyl alcohol might be metabolized via downstream 5-hydroxylation and subsequent O-methylation. Besides the decrease in the concentration of compounds derived from sinapate and ferulate, BnREF1i seeds of line 29b displayed a remarkable reduction in the content of metabolites (18 and 60) derived from 5-hydroxy ferulate. This indicates a reduced formation of 5-hydroxy ferulate from 5-hydroxy coniferaldehyde, thereby supporting the hypothesis that the BnREF1 encoded-CALDH/SALDH might also convert 5-hydroxy coniferaldehyde into the corresponding hydroxycinnamate 5-hydroxy ferulate. In analogy, the reduced level of the cyclic bis-isofuranyl spermidine conjugate (39) indicates that the BnREF1i encoded CALDH/SALDH might also be involved in the biosynthesis of isofurulate. However, these putative enzymatic activities remain to be characterized by in vitro assays.

More upstream in the phenylpropanoid pathway, we detected significant changes in the fraction of kaempferol glycosides. Here, decreasing amounts of sinapoylated compounds (46–48) reflected the restricted supply of sinapate moieties. On the other hand, an increase was observed in the amounts of nonacylated kaempferol 3-O-b-S-coupled sinapoyl alcohol (43). The overaccumulation of compounds 42 and 43 was also found in the oilseed rape transformant suppressing UDP-Glc: sinapate glucosyltransferase (UGT84A9), the enzyme converting sinapate to 1-O-sinapoyl-glucose (Wolfram et al., 2010) and in the transgenic over-expressor of sinapoylcholine esterase (BnSCE3) involved in sinapine hydrolysis (Clauss et al., 2011). Hence, this metabolic change should be related to a perturbed sinapate ester metabolism.

Engineering BnREF1: A Tool to Generate Low-Sinapine Oilseed Rape?

Sinapate conjugates account for the vast majority of phenolic compounds in seeds of oilseed rape. Therefore, a successful approach to generate lines with a
decreased phenolic content designated as “low-sinapine trait” should ideally suppress the biosynthesis of sinapate without affecting other metabolic steps that are required for plant vigor and performance. According to its metabolic function in the phenylpropanoid pathway (Fig. 1), the \textit{REF1}-encoded aldehyde dehydrogenase \textit{Caldh/Saldh} was predicted to meet these requirements (Nair et al., 2004). The \textit{REF1}-suppressing oilseed rape line 29b produced seeds with a sinapate content of about 42% and a total sinapate ester content of about 35% compared with the nontransformed wild type. This was in the range reported for the suppression of UGT84A9 (Hüsken et al., 2005; Wolfram et al., 2010). Although efficient in reducing sinapate ester...
content, the silencing approaches reported so far could not outperform the recently demonstrated overexpression of sinapine esterase BnSCE3. BnSCE3-overexpressing seeds were nearly devoid of sinapine. However, in contrast to the silencing of BnREF1 and UGT84A9, the fraction of related sinapate esters was not affected by the induced sinapine esterase activity (Clauss et al., 2011). With regard to conventional breeding, BnREF1 genes appear as the favored molecular targets. Unlike BnSCE3 overexpression, BnREF1 suppression could be established by nontransgenic mutagenesis approaches. In contrast to UGT84A9, CALDH/SALDH is encoded by only two single-copy homologous genes, BnREF1-I and BnREF1-II, and overlapping enzyme activities are not known. As an indispensable requirement for a potential use in molecular breeding, the overall performance of oilseed rape transformatants that suppress BnREF1 constitutively should be evaluated. Although the Arabidopsis ref1 mutant seems not impaired, the expected constitutive decrease in leaf sinapoylmalate content and cell wall-associated phenolics bears the potential to increase the sensitivity of BnREF1-deficient plants against UV-B and pathogen stress.

MATERIALS AND METHODS

Plant Material and Cultivation

Winter oilseed rape (Brassica napus var napus ‘Express’), forage kale (Brassica oleracea var medullosa ‘Markola’), and turnip (Brassica rapa var campestris ‘Rex’) were obtained from Nordeutsche Pflanzenzucht. Plants were grown in the greenhouse at 12°C to 18°C under a 16-h light regimen.

Nucleic Acid Techniques

Purification of DNA and RNA was done by selective adsorption onto silica using commercial preparation kits (Qiagen; http://www.qiagen.com). To prevent DNA contamination of RNA preparations, on-column digestion with DNase I was included in the extraction protocol of total RNA. Poly(A)^+ RNA was enriched from total RNA by selective binding to oligo(dT)-Oligotex beads (Qiagen). For RT-PCR, total RNA (1 μg) was reverse transcribed in a 20-μL reaction mixture using the Omniscript RT kit (Qiagen) and an oligo-(dT)15 primer (Promega; http://www.promega.com). The RT reaction mix was diluted 25-fold and included as template into a 20-μL PCR mixture (MangoMix; http://www.bioline.com) with appropriate primers. PCR-based techniques were performed according to protocols given by the commercial suppliers of reaction mixtures. The primers used are listed in Supplemental Table S1. Molecular cloning and restriction analyses were performed according to standard protocols (Sambrook et al., 1989). DNA sequencing was done by a commercial supplier (Eurofins MWG Operon).

Cloning of BnREF1 Genes

Sequence information of Arabidopsis (Arabidopsis thaliana) REF1 (At3g24503) and related ESTs from oilseed rape (GenBank accession nos. CD811437, CD828462, CD838287, and CD839291) was used to generate primers 1 and 2 (Supplemental Table S1) for PCR amplification of a BnREF1-cDNA subfragment. cDNA generated from oilseed rape seeds at the well-developed mature embryo stage (stage E; Milkowski et al., 2004) was used as the PCR template. PCR was run with AccuPrime™ DNA Polymerase (Invitrogen; http://www.invitrogen.com). PCR amplification products were cloned into plasmid pCR-BluntII-TOPO (Invitrogen), transformed into E. coli strain Top10 (Invitrogen), and evaluated by sequence analysis. BnREF1 genomic sequences were PCR amplified with primer pair 5 and 6 (Supplemental Table S1) and genomic DNA isolated from young leaves of oilseed rape as template. PCR was run with AccuPrime™ DNA Polymerase (Invitrogen). Amplification products were cloned into plasmid pCR-BluntII-TOPO (Invitrogen), transformed into E. coli strain Top10 (Invitrogen), and subjected to sequence analysis.

Southern Blot

Five micrograms of genomic DNA isolated from young leaves of oilseed rape, kale, and turnip was digested overnight with the appropriate restriction endonuclease in a volume of 100 μL. DNA fragments were separated by electrophoresis run for 20 h in 0.4% Tris-acetate and 0.001% EDTA, pH 8.0, using a 0.75% agarose gel. Digoxigenin (DIG)-labeled DNA M, Marker II (http://www.roche-applied-science.com) was applied for size determination. Blotting onto a nylon membrane (Hybond NX; Amersham Biosciences; http://www.gelifesciences.com) and hybridization were carried out as described previously (Mittasch et al., 2010). The BnREF1 probe was PCR labeled with primers 9 and 10 (Supplemental Table S1) and BnREF1-cDNA as template using the PCR DIG Probe Synthesis kit (Roche). The amplified DIG-labeled BnREF1 probe covered 419 nucleotides, including sequences from exons 2 and 3 (Fig. 2). Hybridization signals were visualized with the DIG Luminescent Detection kit (Roche).

Construction of the BnREF1 Suppression Vector pLH-BnREF1i

A subfragment of BnREF1-cDNA covering 419 bp of the reading frame (nucleotide positions 128-546) was amplified by PCR using the primer pair 9 and 10 (Supplemental Table S1). The PCR product was 5′ flanked by restriction sites for NheI and Ncol and 3′ flanked by restriction sites for Smal and BamHI. This structure allowed us to assemble a hairpin suppression cassette consisting of the 419-bp BnREF1 fragment in the antisense orientation (as Smal-Ncol fragment), a subfragment of the bacterial uidA gene designated as GUS (Chuang and Meyerowitz, 2000) as spacer element, and the BnREF1 fragment in the sense orientation (as NheI-BamHI fragment). This double-stranded DNA interference cassette was inserted between the seed-specific napin promoter and the nos terminator. The seed-specific napin promoter covers nucleotides 1,594 to 2,182 of the NAPINC gene from oilseed rape (GenBank accession no. M66433) encoding a seed storage protein of the napin type. Seed specificity of this promoter has been demonstrated (Krill et al., 1991), and the promoter has been used frequently for seed-specific transgenic approaches in oilseed rape (Hüskens et al., 2005; Clauss et al., 2011). Assembly of these elements was done in plasmid pBlueScript II KS−, from which the whole suppression construct was cut as SpeI-HindIII fragment and inserted into the binary vector pLH7000 (Hausmann and Töpfer, 1999) to give the seed-specific BnREF1 suppression plasmid pLH-BnREF1i.

Transformation of Oilseed Rape

Binary plasmids (pLH7000, pLH-BnREF1i) were used to transform Agrobacterium tumefaciens strain C58C1 (Hellens et al., 2000). A. tumefaciens-mediated transformation of oilseed rape ‘Lisora’, plant regeneration, and selection for PPT resistance were performed as described previously (Hüskens et al., 2005). Resistant plants regenerated from PPT-resistant calli were designated as the first transgenic generation (T1). T1 plants were grown under greenhouse conditions and selfed to produce T2 seeds. After germination, T2 seeds gave rise to T2 plants, which were grown and selfed to produce T3 seeds. Descendant transgenic generations were propagated accordingly. Mature dry seeds were harvested and stored at 20°C.
Genotyping of BnREF1i Transformants

Genomic integration of the BnREF1i suppression cassette was proven by PCR with 0.1 ng of genomic DNA from oilseed rape transformants. After initial denaturation (2 min, 94°C), PCR was run for 30 cycles (10 s, 94°C; 30 s, 55°C; 40 s, 72°C) with Taq DNA polymerase included in a commercially available master mix (BioMix Red; Bioline). Primers 11 and 12 (Supplementary Table S1) binding to sequences within the napin promoter and the GUS spacer region of the transgene cassette were used to amplify a 658-bp DNA fragment that covers the antisense arm of BnREF1i. Primers 13 and 14 (Supplementary Table S1) recognizing sequence motifs within the GUS spacer and the nos terminator were employed to amplify a 704-bp fragment containing the sense arm of BnREF1i. Genotyping of the T4 generation was done with the Phire Plant Direct PCR Kit, Finnzymes (http://www.thermoscientificbio.com/finzymes/). The manufacturer’s instructions were followed, and primer annealing temperatures were calculated using the melting temperature calculator offered by Finnzymes (http://www.thermoscientificbio.com/webtools/mtm/).

Sequence Analysis

DNA and protein sequences were analyzed by the software package Clone Manager and the Sequencing Analysis Software. BLAST algorithm (Altschul et al., 1990) was employed. Online databases used were GenBank (http://www.ncbi.nlm.nih.gov/GenBank/index.html) and The Arabidopsis Information Resource (http://www.arabidopsis.org/).

Expression of BnREF1 in E. coli

A full-length BnREF1 coding sequence except for the start codon was amplified from BnREF1i cDNA by PCR with primers 7 and 8 (Supplementary Table S1) introducing restriction sites for BamHI and SalI using AccuPrimePfx DNA Polymerase (Invitrogen). The amplification product was inserted as BamHI-SalI fragment into plasmid pQE30 (Qiagen). The ligation product was transformed into E. coli strain M15 [pREP4] (Qiagen). For heterologous expression, cells were grown under vigorous shaking at 37°C in liquid Luria-Bertani medium supplemented with ampicillin (100 μg mL⁻¹) to the early exponential phase. The expression of BnREF1i was then induced by further cultivation in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside for 2 h. Cells were harvested by centrifugation (10 min, 10,000g, 4°C), and the cell pellets were stored at -80°C.

Caldh/Saldh Activity Assays

Aldehyde dehydrogenase activity toward coniferaldehyde and sinapaldehyde was assayed as described previously (Nair et al., 2004). For protein extraction from E. coli expressing BnREF1i, the cell pellets were resuspended in reaction buffer containing 50 mM HEPES-KOH, pH 8.0, 1 mM EDTA, 5 mM dithiothreitol, and 10% (v/v) glycerol. Cells were lysed by sonication, and the soluble protein was separated from the cell debris by centrifugation (20 min, 10,000g, 4°C). Protein extraction from seeds of oilseed rape was done by grinding the seeds (1 g) in liquid nitrogen. The powder was added to 10 mL of ice-cold reaction buffer, and the suspension was stirred at 4°C for 30 min. After centrifugation (20 min, 10,000g, 4°C), the protein was precipitated from the supernatant by adding ammonium sulfate to 80% saturation. After centrifugation, the pellets were redissolved in reaction buffer and the solutions were desalted using PD-10 Sephadex G-25 columns (Amersham). Protein contents were determined by the method of Bradford (1976) with bovine serum albumin as the standard. Aldehyde dehydrogenase assays were performed in a final volume of 150 μL of reaction buffer containing 1 mM NAD⁺, 100 μM coniferaldehyde or sinapaldehyde, and 50 μL of the soluble protein extract. Reactions were started by adding the protein to the substrates. Reaction mixtures were incubated at 30°C for 60 min. CALDH and SALDH enzyme activities were calculated from the increase in the formation of reaction products, ferulate and sinapate. The formation of these reaction products was analyzed by reverse-phase HPLC. Ferulate and sinapate were quantified by external standardization with reference compounds.

Quantification of Sinapate Esters in Single Seeds and Seed Bulks

For single-seed analyses, the seeds (T2 and wild type) were ground individually and extracted two times with 250 μL of 80% (v/v) aqueous methanol solution in 1.5-mL safe-lock tubes in the presence of 1.0-mm zirconia beads using a bead beater (both from BIOCSPC Products; http://www.bioesp.com). Extracts were centrifuged for 10 min at 16,000g; Sinapate and sinapoylglycine were quantified directly in extracts after dilution (1:1, v/v) with water. The total sinapate ester content was calculated from the amount of sinapate determined after saponification of extracts. For alkaline hydrolysis, 50 μL of extract was treated with 50 μL of 2 M KOH for 3 h at 50°C. The reaction was quenched by adding 50 μL of 25% (v/v) aqueous acetic acid. Quantification of sinapate, sinapoylglycine, and sinapate was performed by external calibration using authentic standards. The separations were performed on a Waters BEH C18 column (2 × 50 mm, particle size of 1.7 μm) using a Waters Acuity UPLC System equipped with an Acquity Binary Solvent Manager, Acquity Sample Manager, and 2-mL sample loop, partial injection mode (200 μL injection volume), and Acquity PDA Detector and controlled by Empower 2 Software. Eluent A and B were water and 98% (v/v) aqueous acetonitrile, respectively, both containing 0.1% (v/v) triﬂuoroacetic acid. After isocratic elution for 0.5 min at 5% eluent B, linear gradients to 27% and 95% eluent B in 1.3 and 0.2 min were started. The flow rate and column temperature were set to 500 μL min⁻¹ and 40°C, respectively. Phenylpropanoids were detected in photodiode array mode at 220 to 490 nm and in single-wavelength mode at 330 nm.

For bulk analyses of sinapate esters, 20 seeds (T3 and wild type) were ground in liquid nitrogen to a fine powder using a pestle and mortar. After lyophilization, aliquots of 10 ± 2 mg were extracted two times with 750 μL of an 80% (v/v) aqueous methanol solution by sonication for 10 min at 20°C. Cell debris were pelleted by centrifugation for 10 min at 16,000g, and the supernatant was used for HPLC-based determination of sinapate and total sinapate contents after saponification. For each of the transgenic lines (27a and 29b) and the wild-type control (oilseed rape ‘Lisora’), seed pools from three individual plants (biological replicates) were analyzed in duplicate (technical replicates).

Sample Preparation for Metabolite Profiling

Subsamples (1.5 g) of seed pools (T4 and wild type) were roughly ground in liquid nitrogen using a pestle and mortar, and aliquots of 50 ± 2 mg were weighed into precooled 2-mL polypropylene tubes containing three steel balls (diameter of 2 mm). After the addition of 1,000 μL of 50% (v/v) aqueous methanol, which was precooled to -80°C and spiked with phlorizin (20 μM) and anisic acid (20 μM) as internal standards, the samples were homogenized for 3 min at 30 Hz using a mixer mill (MM301; http://www.retsch.de). After sonication for 15 min at 20°C, the samples were centrifuged for 10 min at 16,000g. For the preparation of a total extract (2.5 mg fresh weight mL⁻¹ extract), 30 μL of supernatant was diluted with 950 μL of water and filtered using syringe filters (0.2 μm, polytetrafluoroethylene; Whatman; http://www.whatman.com). For the preparation of choline ester-depleted extracts (25 mg fresh weight mL⁻¹ extract), 100 μL of supernatant was diluted with 300 μL of water and subjected to solid-phase extraction on a weak cation-exchange polymeric sorbent. Therefore, Strata-X-CW columns (30 mg; Phenomenex; http://www.phenomenex.com) were solvated with 1 mL of methanol and equilibrated with the same amount of water. After sample loading, the columns were washed with 500 μL of ammonium acetate (25 mM) and 1 mL of methanol. Fractions obtained during loading and washing steps were combined and evaporated to dryness under reduced pressure at 40°C using a vacuum centrifuge. The remaining residues were thoroughly reconstituted in 200 μL of 10% (v/v) aqueous methanol, sonicated for 10 min at 20°C, and filtered using syringe filters (0.2 μm, polytetrafluoroethylene; Whatman).
UPLC/ESI-QTOFMS

Chromatographic separations were performed on an Acquity UPLC system (Waters) equipped with a high-sensitivity silica T3 column (100 × 1.0 mm, particle size of 1.8 μm; Waters) applying the following binary gradient at a flow rate of 150 μL min⁻¹: 0 to 1 min, isocratic 95% A (0.1% [v/v] formic acid in water), 5% B (0.1% [v/v] formic acid in acetonitrile); 1 to 10 min, linear from 5% to 40% B; 10 to 12 min, isocratic 95% B; 12 to 14 min, isocratic 5% B. The injection volume was 3.1 μL (full-loop injection). Eluted compounds were injected from mass-to-charge ratio (m/z) 90 to 1,300 using a microQTOF-Q II hybrid quadrupole time-of-flight mass spectrometer (Bruker Daltonics; http://www.bruker.com) equipped with an Apollo II electrospray ion source in positive and negative ion mode using the following instrument settings: nebulizer gas, nitrogen, 1.4 bar; dry gas, nitrogen, 6 L min⁻¹; curtain, 5.000 V (+4,000 V); end plate offset, −500 V; funnel 1 radio frequency (RF), 200 V; funnel 2 RF, 200 V; in-source collision-induced dissociation (CID) energy, 0 V; hexapole RF, 100 V; quadrupole ion energy, 5 eV; collision gas, nitrogen; collision energy, 7 eV; collision RF, 150/350 V (timings, 50/50); transfer time, 70 μs; prepulse storage, 5 μs; pulser frequency, 10 kHz; spectrometer, 3 Hz. Mass spectra were acquired in centroid mode. Calibration of the instrument was performed for individual raw data files. Stability of experiments was confirmed by an analysis of elemental compositions of fragment ions calculated from accurate m/z of CID mass spectra. Compounds were then putatively annotated/characterized by interpretation of characteristic fragment ions and neutral losses (Supplemental Tables S2 and S3). Putative annotations of a total of 26 compounds could be verified by an analysis of noncommercial reference compounds that were synthesized (Böttcher et al., 2009) or isolated from oilseed rape seeds and structurally characterized by NMR (Baumert et al., 2005; Wolfram et al., 2010) in previous studies.

The isolated genomic sequences described in this paper have been submitted to the EMBL Nucleotide Sequence Database and assigned the accession numbers FN995990 (BinREF1_I) and FN995991 (BinREF1_H).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Transcript abundance of BinREF1 in oilseed rape revealed by electronic northern-blot analysis.

Supplemental Figure S2. Transcript abundance of BinREF1 in seeds of transgenic lines 27a and 29b of oilseed rape compared with the non-transformed wild type.

Supplemental Figure S3. Molecular structures of annotated metabolites.

Supplemental Figure S4. Heat-map representation of relative quantification of the annotated metabolites.

Supplemental Table S1. Oligonucleotides used as PCR primers.

Supplemental Table S2. Analytical data of annotated compounds.

Supplemental Table S3. Mass spectral data of annotated compounds.

Supplemental Data S1. Relative quantification of the annotated metabolites and statistical analyses.

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