Identification of differentially methylated regions during vernalization revealed a role for RNA methyltransferases in bolting

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Abstract

Sugar beet (Beta vulgaris altissima) is a biennial root crop with an absolute requirement for cold exposure to bolt and flower, a process called vernalization. Global DNA methylation variations have been reported during vernalization in several plants. However, few genes targeted by DNA methylation during vernalization have been described. The objectives of this study were to identify differentially methylated regions and to study their involvement in bolting induction and tolerance. Restriction landmark genome scanning was applied to DNA from shoot apical meristems of sugar beet genotypes, providing a direct quantitative epigenetic assessment of several CG methylated genes without prior knowledge of gene sequence. Several differentially methylated regions exhibiting variations of gene-body DNA methylation and expression during cold exposure and/or between genotypes were identified, including an AROGENATE DEHYDRATASE and two RNA METHYLCYTOSINE TRANSFERASE sequences. One RNA METHYLCYTOSINE TRANSFERASE sequence displayed gene-body hypermethylation and activation of expression, while the other was hypomethylated and inhibited by cold exposure. Global RNA methylation and phenolic compound levels changed during cold exposure in a genotype-dependent way. The use of methyl RNA immunoprecipitation of total RNA and reverse transcription–PCR analysis revealed mRNA methylation in a vernalized bolting-resistant genotype for the FLOWERING LOCUS 1 gene, a repressor of flowering. Finally, Arabidopsis mutants for RNA METHYLCYTOSINE TRANSFERASE and AROGENATE DEHYDRATASE were shown to exhibit, under different environmental conditions, early or late bolting phenotypes, respectively. Overall, the data identified functional targets of DNA methylation during vernalization in sugar beet, and it is proposed that RNA methylation and phenolic compounds play a role in the floral transition.

Key words: Arabidopsis thaliana mutant, bolting tolerance, differentially methylated region, DNA methylation, epigenetic, RNA methylation, sugar beet, vernalization.

Introduction

Sugar beet (Beta vulgaris altissima) is a biennial root crop providing 30% of the world’s sugar. Bolting and flowering in this long-day species require a prolonged exposure to cold temperatures between 2 °C and 10 °C (Lexander, 1980) called...
Vernalization. Vernalized sugar beet plants can revert to a vegetative state under short-day conditions or under high temperature following vernalization, a phenomenon called devernalization (Lexander, 1980; Smit, 1983). Sugar beet bolting corresponds to a rapid elongation of the stem associated with the use of stored sucrose, and is usually followed by the development of an indeterminate inflorescence. It is widely accepted that this plant can bolt without flowering, but rarely flower without bolting (Mutusza-Gottgens et al., 2009). Cold temperatures in spring can mimic winter vernalization and induce bolting. The expected global climate change should affect bolting of beet crops (Perarnaud et al., 2001) and allow earlier sowing (Thomas, 2008). The annual habit in beets was shown to be under the genetic control of a dominant Mendelian factor termed B also called ‘bolting gene’, which overrides the need for vernalization by promoting flowering (Abegg, 1936). Thus annual beets (BB or Bb) are non-responsive to vernalization, while biennial beets (bb) are responsive to vernalization. Bolting tolerance is a quantitative agronomic trait reflecting the bolting response of biennial genotypes after a cold exposure. Several parameters are used to measure the bolting tolerance, such as the bolting index (BI) corresponding to the percentage of bolting plants, and the bolting delay (BD), which is the average number of days required for a visible bolting initiation. Breeders still largely depend on phenotypic rather than genotypic selection, and the mechanisms controlling the variation in bolting tolerance between different beet genotypes have still not been elucidated.

The genetic control of flowering was first elucidated in the annual model plant Arabidopsis thaliana. It includes several regulatory pathways that differ in their response to distinct cues such as vernalization, photoperiod, and autonomous pathways (Baûre and Dean, 2006). Vernalization requirement and response in Arabidopsis is centrally regulated by the repressor of flowering FLOWERING LOCUS C (FLC), a MADS box gene. The expression of FLC is down-regulated by the vernalization and autonomous pathways leading to the activation of bolting and flowering (Kim et al., 2009). In recent years, many studies have demonstrated that vernalization in Arabidopsis is controlled by epigenetic mechanisms. Epigenetic marks can be transmitted to daughter cells by mitosis, as is the case for the vernalization process in Arabidopsis which relies on the somatic transmission of cold-induced repressive chromatin over FLC (Roudier et al., 2009). Indeed, vernalization is associated with a small, transient decrease in DNA methylation in Arabidopsis (Burn et al., 1993; Finnegan et al., 1998) and FLC is down-regulated in plants where the level of DNA methylation has been reduced by an antisense construct against the DNA METHYLTRANSFERASE 1 (MET1; Sheldon et al., 1999). However, FLC is not directly regulated by DNA methylation (Finnegan et al., 2005). Although there are similarities between the response of FLC to vernalization and to low DNA methylation, these two pathways inducing the chromatin-based epigenetic control of FLC activity appear to be distinct (Finnegan et al., 2005).

In sugar beet, several orthologues of genes involved in the photoperiod, autonomous, and vernalization pathways have been identified recently (Reeves et al., 2007; Chia et al., 2008; Pin et al., 2010; Abou-EIwafa et al., 2011; Trap-Gentil et al., 2011). Both partial conservation and divergence in the genetic basis of bolting and flowering were found between sugar beet and Arabidopsis. For example, as in Arabidopsis, the sugar beet homologue BvFL1 is down-regulated in response to cold, but, unlike AtFLC, its repression is not maintained after vernalization (Reeves et al., 2007). In addition, two paralogues of the Arabidopsis FLOWERING LOCUS T (FT) gene, named BvFT1 and BvFT2, were isolated, mapped on sugar beet chromosomes, and shown to have evolved antagonistic functions (Pin et al., 2010). BvFT2 is the functional FT orthologue and promotes flowering, while BvFT1 represses flowering and its down-regulation is necessary for the vernalization response. BvFT1 is also important for the devernalization phenomenon. The recent map-based cloning of the bolting locus B revealed the presence of several genes (Pin et al., 2012). One corresponds to a pseudo-response regulator gene called BOLTING TIME CONTROL 1 (BvBTC1) which acts as an upstream regulator of BvFT1 and BvFT2 and is up-regulated by long days in annuals. The rare recessive allele Bvbtc1 in biennial beet cannot transduce the sensitivity to photoperiod but retained a role as a promoter of bolting under vernalization. Taken together, these data suggested that the vernalization response has evolved different mechanisms not only in monocots such as cereals (Dennis and Peacock, 2009) and eudicots (Schmitz and Amasino, 2007), but also between different eudicots (Pin et al., 2010, 2012). Nevertheless, the vernalization gene network and the mechanisms controlling the expression of all these sugar beet orthologues are still poorly known. Due to unexpected experimental observations, such as the bolting of a subset of Bvbtc1 or BvFT2 RNAi (RNA interference) plants after vernalization, additional mechanisms must exist to promote bolting in sugar beet (Pin et al., 2010, 2012). According to this, an epigenetic control of vernalization has recently been observed in sugar beet (Trap-Gentil et al., 2011). Treatment leading to DNA hypermethylation was shown to inhibit and delay bolting. In addition, vernalization and devernalization affected DNA methylation levels in the shoot apical meristem of several sugar beet genotypes. These variations could be negatively correlated with their bolting tolerance. Indeed, genotypes with different bolting tolerance levels could be distinguished by their global DNA methylation levels in shoot apical meristem as well as by their methylation patterns at the BvFL1 locus, providing possible new tools for the selection of bolting tolerance (Trap-Gentil et al., 2011). The changes in DNA methylation during vernalization could take part in the control of genes involved in bolting release or in the bolting tolerance level.

The objectives of this study were to identify sequences targeted by changes in DNA methylation during vernalization and between sugar beet genotypes using a genome-scanning approach, and to study their possible involvement in bolting induction and tolerance between genotypes. Several differentially methylated regions (DMRs) exhibiting variations of gene-body DNA methylation and expression during cold exposure and/or between genotypes were identified. Among
them, an AROGENATE DEHYDRATASE (ADT) and two putative RNA METHYLCYTOSINE TRANSFERASE (RNMT) sequences were studied further; one RNMT was hypermethylated and activated by cold exposure while the other was hypomethylated and inhibited. The total RNA methylation and phenolic compound levels were also affected by cold exposure in a genotype-dependent way. Interestingly, methyl RNA immunoprecipitation (MeRIP) and reverse transcription–PCR (RT–PCR) analysis showed that the mRNA of the flowering repressor BvFL1 was both up-regulated and methylated after cold exposure in shoot apical meristems of a bolting-resistant genotype. Finally, Arabidopsis mutants for ADT or RNMT displayed either early or late bolting/flowering under different environmental conditions, respectively. Altogether, the data identified functional targets of DNA methylation in the shoot apical meristem during vernalization, and a role for RNA methylation and phenolic compounds in the floral transition was proposed.

Materials and methods

Plant materials, growth conditions, and treatments

Eleven biennial sugar beet genotypes (B. vulgaris altissima) provided by SES-VanderHave, Tienen, Belgium) were germinated and grown for 8 weeks at 22 °C under a 16 h photoperiod (700 µmol m⁻² s⁻¹). Vernalization was at 4 °C for 0, 3, 15, or 18 weeks, as described by Trap-Gentil et al. (2011). For each genotype and treatment duration, 24 plants were sampled as follows: shoot apical meristems of 14 plants were immediately collected, dissected away from differential tissues to isolate the shoot apical meristem (dotted line in Supplementary Fig. S1 available at JXB online), frozen in liquid nitrogen, and stored at −80 °C until used. The 10 remaining plants were placed for 6 weeks at 22 °C under optimal light conditions (1000 µmol m⁻² s⁻¹). During this period, two bolting parameters were recorded daily: bolting index (BI), which is the percentage of bolting plants, and bolting delay (BD), which is the average number of days required for a visible bolting initiation. Genotypes S1–S6 (previously named G2, G1, G3, G9, G12, and G16, respectively, in Trap-Gentil et al. 2011) started to bolt after 9 weeks at 4 °C and reached 100% BI at the end of the treatment with an average BD value of 25 d, and are considered as sensitive (S) to bolting. Genotypes R1–R5 (previously named G5, G6, G4, G10, and G7, respectively) are classified as resistant (R) with 60% BI and an average BD value of >30 d.

Arabidopsis (Arabidopsis thaliana) seed stocks were obtained from the Nottingham Arabidopsis Stock Centre (Loughborough, UK): wild-type Columbia (N60000); SALK_026873 and SALK_041016 which are T-DNA knockout at adt loci; SALK_041016 and SM_3.20773, which are T-DNA knockout at adt. ADT (At1g11790) and RNMT (At3g13180) encode an AROGENATE/PREPHRENATE DEHYDRATASE and a RNA METHYLTRANSFERASE, respectively. Sequences were retrieved from the Genbank database (http://www.ncbi.nlm.nih.gov/). The T-DNA sequences were screened using the green plant protein database from Phytozyme (http://www.phytozyme.net/search.php?show=blast&method). Multiple alignments for protein and nucleotide sequences and phylogenetic trees were performed with CLUSTAL 2.0.10 multiple sequence alignment (http://www.ebi.ac.uk/Tools/clustalw2). The identification of potential transposable elements (TEs) in the RLGS genomic sequences was realized by the identification of potential coding sequences (FGENESH on http://linux1.softberry.com/berry.phtml) followed by BLASTP analysis and the search of dispersed genomic repeats using PLOTREP (http://repeat.ahe.bidi/cgi-bin/plotrep.pl). Computational analysis of microRNA (miRNA) targets was also done using psRNA2Target (http://bioinfo3.noble.org/psRNA2Target/index.php?function¼function2). Expression and methylation patterns for RNMT and ADT (At3g13180 and At1g11790, respectively) were obtained using microarray analysis of the Arabidopsis developmental map (Arabidopsis eFP browser, http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter et al., 2007) and Arabidopsis methylome data (http://neomorph.salk.edu/epigenome/epigenome.html; Zhang et al., 2006).

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Restriction landmark genome scanning (RLGS) method for methylation and cloning

Genomic DNA was purified individually from each sugar beet shoot apical meristem after 0, 3, 15, or 18 weeks of vernalizing treatment in a very sensitive very early bolting sugar beet genotype S1 (100% BI and average BD of 21.4 d) and a resistant late bolting genotype R1 (70% BI and average BD of 28.5 d). An already published DNA extraction protocol (Causevic et al., 2005) was applied using extraction buffer containing 0.2 M TRIS-HCl and 0.2 M EDTA (pH 9.0), supplemented with 1.5% SDS and 10 mM β-mercaptoethanol. Then, an RNase A digestion step (complete removal of RNAs), phenol/ chloroform extraction, and ethanol precipitation were performed. The RLGS protocol and the cloning of the RLGS fragments were performed according to Causevic et al. (2006). Sequencing (four cycles by marker) was performed using a Big-Dye Terminator v3.1 sequencing kit (Applied Biosystems, Courtaboeuf, France) and carried out with an ABI 3100 automated sequencer (Applied Biosystems, Carlsbad, CA, USA). The GenBank accession numbers of the RLGS markers, numbered from 1 to 39, are HR807120–HR807158, respectively.

Bioinformatic analyses

Potential CpG islands in the cloned RLGS sequences were analysed using ‘CpgIsland Searcher’ software (http://cpgislands.usc.edu/; Takai and Jones, 2002). Open reading frame (ORF) identification was performed using ‘genscan’ (http://mobyle.pasteur.fr/cgi-bin/portal.py#forms:genscan) and ‘ORF Finder’ (http://www.bioinformatics.org/sms/orf/find.html). Blast analyses (TBLASTX and BLASTN) were performed using the flowering plants genomic and expressed sequence lag (EST) databases from the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the TIGR Plant Transcript Assembly (http://blast.jcvi.org/euk-blast/plantta_blast.cgi). BLASTP analyses were performed using the green plants protein database from Phytozyme (http://www.phytozyme.net/search.php?show=blast&method).
Methyl-sensitive PCR

Methyl-sensitive PCR (MS-PCR) using the MorBC enzyme was adapted from Trap-Gentil et al. (2011). The BrFL1 sequence (EF036526) corresponding to a FLOWERING LOCUS C-LIKE gene in sugar beet and a MITOCHONDRIAL CYTOCHROME OXIDASE SUBUNIT I gene (DQ381450) named BrCYT were used as hyper- and hypomethylated controls, respectively (Trap-Gentil et al., 2011). Primers for markers 2, 18, 23, 32, and 37 were designed with ‘Eprimer3’ software (http://emboj.bininformatics.nl/cgi-bin/emboj/epprimer3) and are described in Supplementary Table S1 at JXB online. For the markers 23 and 32, two primer pairs for each were designed on regions showing nucleotide polymorphism to allow them to be distinguished. Two biological repeats were analysed in triplicate for each genotype and duration of treatment.

Bisulphite sequencing

Bisulphite treatment was applied to determine the cytosine methylation status using the Epitect bisulphite kit (Qiagen) and 500 ng of genomic DNA. Controls for bisulphite conversion or amplification, primer design, PCR conditions, sequencing, and quantification of cytosine methylation percentages have been recently detailed in Trap-Gentil et al. (2011). Primers for BrATD (18), BrRMF1a (23), and BrRMF1b (32) are described in Supplementary Table S1 at JXB online. A methylcytosine signal below 10% was interpreted as a hypomethylated position (cytosine), a signal >90% as a hypermethylated position (methylcytosine), and an intermediate signal (10–90%) was classified as a mixture of cytosine and methylcytosine.

RNA extraction and semi-quantitative RT–PCR analysis

Total RNAs were isolated from sugar beet shoot apical meristems of sugar beet or Arabidopsis plants using Nucleospin® RNA Plant (Macherey-Nagel, Hoerdt, France) and reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems). Internal standards to normalize the amount of mRNA in the PCR, controls, and PCR conditions were according to Trap-Gentil et al. (2011). Primers were identical to those designed for MS-PCR amplification of sugar beet sequences (see Supplementary Table S1 at JXB online).

Determination of global RNA methylation percentages by HPLC

A 5 µg aliquot of total RNAs extracted from shoot apical meristems of sugar beet or Arabidopsis plants was digested successively with 7 U of RNase A (Sigma-Aldrich) at 37 °C for 1.5 h, 0.05 U of phosphodiesterase I (USB, OH, USA) at room temperature for 1.5 h, and 0.5 U of alkaline phosphatase type III (Sigma-Aldrich) at room temperature for 2.5 h. After precipitation with 2 vols of 96% cold ethanol to eliminate undigested RNAs, the supernatant containing ribonucleosides was vacuum dried, resuspended in ultrapure water, and injected onto a hydrophobic Gemini™ C18 column (150×4.6 mm, 5 µm, Phenomenex, Le Pecq, France). The isocratic mobile phase was water/methanol (199:1 v/v) supplemented with 0.03% acetic acid (v/v) at 2 ml min⁻¹ flow rate for 35 min. Identification of cytidine (C) and methylcytidine (MC) were assessed by co-migration with commercial standards (Sigma-Aldrich) under the same HPLC conditions. The methylcytidine percentages were calculated using the following formula: %MC=[(MC/(C+MC))/[MC0/(C0+MC0)]×100 for sugar beet (S0 corresponds to sensitive genotypes before vernalization) and %MC=[(MC/(C+MC))/[MC0/(C0+MC0)]×100 (Col-0 corresponds to the wild type) for Arabidopsis. Three independent biological and three technical repeats were performed for each analysis.

Methyl RNA immunoprecipitation (MeRIP) and semi-quantitative RT–PCR

Total RNAs extracted from a bolting-resistant genotype (10 µg; see RNA extraction section above) were diluted in the immunoprecipitation buffer (10 mM Na-phosphate pH 7.0 supplemented with 0.14 M NaCl and 0.05% Triton X-100). An aliquot of total RNA (4 µg) was stored at −80 °C and corresponds to an input control. Immunoprecipitation of methylated RNA was performed with the remaining RNA (6 µg) according to Weng et al. (2009) using 10 µg of 5-methylcytidine monoclonal antibody (Eurogentec, Angers, France). Two successive RNA extractions were then carried out using phenol/chloroform/isoamyl alcohol (25/24/1) and chloroform/isoamyl alcohol (24/1). RNAs were precipitated at −80 °C during 30 min after addition of 96% ethanol (2.5 vols) and 3 M Na-acetate pH 5.2 (0.1 vol.). RNAs were washed using 70% ethanol, vacuum dried, and resuspended in RNase-free water. Immunoprecipitated and total RNAs were analysed by semi-quantitative RT–PCR as described above. The number of cycles was adapted to each RNA fraction to avoid reaching saturation and to obtain similar amplification for housekeeping genes between immunoprecipitated and total RNAs. Primers for BrTUBULIN, BrUBIQUITIN, the Beta vulgaris FLOWERING LOCUS T-LIKE1 and 2 genes (HM448910 and HM448912, respectively), and BrFL1 are listed in Supplementary Table S1 at JXB online.

Extraction of soluble phenolic compounds and HPLC analysis

 Phenolic compounds were isolated from sugar beet shoot apical meristems or from Arabidopsis plants using ice-cold methanol. After centrifugation, methanol extracts were directly injected onto an X-Terra™ C18 column (150×4.6 mm, 5 µm, INTERCHIM, Montluçon, France) for HPLC quantification as described by Causevie et al. (2006). To identify the phenolic compounds, commercial standards such as caffeic, chlorogenic, ferulic, or coumaric acids were also injected. Three biological and three technical repeats were done for each genotype and treatment duration.

Statistical analysis

Statistical analyses were carried out using the SPSS statistical software package (SPSS version 11.0.1 PC, Chicago, IL, USA). Means are expressed with their standard error (±SE) and compared by analysis of variance [ANOVA; General Linear Model (GLM) procedure]. Statistical tests were considered significant at P <0.05.

Results

Identification of differentially methylated regions (DMRs)

Variations in DNA methylation at CpG-rich loci were assessed (Supplementary Fig. S1 at JXB online) by RLGS using the methyl-sensitive NotI enzyme (Supplementary Fig. S1). Shoot apical meristems of a very sensitive very early bolting sugar beet genotype S1 and a resistant late bolting genotype R1 were analysed after different durations of cold exposure (from 0 to 18 weeks; Supplementary Table S2). Eighty-four spots were isolated by RLGS, but only 39 distinct sequences (from 0 to 18 weeks; Supplementary Table S2). Eighty-four spots were isolated by RLGS, but only 39 distinct sequences (from 0 to 18 weeks; Supplementary Table S2). Eighty-four spots were isolated by RLGS, but only 39 distinct sequences (from 0 to 18 weeks; Supplementary Table S2). Eighty-four spots were isolated by RLGS, but only 39 distinct sequences (from 0 to 18 weeks; Supplementary Table S2). Eighty-four spots were isolated by RLGS, but only 39 distinct sequences (from 0 to 18 weeks; Supplementary Table S2).
exposure: four spots were found only in the S genotype (nos 1–4), nine only in the R genotype (nos 16, 17, and 33–39), and one was found for both genotypes but at different locations on the autoradiograms (nos. 5). The RLGS fragments ranged from 121 bp to 859 bp and all contained CpG islands, except markers 10 (which was too short to be analysed according to the definition of CpG islands; Takai and Jones, 2002) and 13. Potential ORFs could be identified for each marker (except marker 13). Results of TBLASTX analyses suggested annotation for five of them (Supplementary Table S2): a potential retrotransposon (BvRTP, marker 2), two enzymes involved in phenylalanine metabolism, an AROGENATE/PREPHRENATE DEHYDRATASE (BvADT, marker 18), and a COUMARATE COENZYME A LIGASE (Bv4CL, marker 37), as well as two RNA METHYLCYTOSINE TRANSFERASE sequences (BvRNMTa and b, markers 23 and 32). Markers 23 and 32 (RNMTa and b) contained an S-adenosylmethionine-dependent methyltransferase superfamily domain and displayed 99% identity for their nucleotide sequences (Supplementary Fig. S3A) while the corresponding translated proteins showed 97% identity (Supplementary Fig. S3B). The sugar beet RNMTs revealed 27–37% identity with RNMT proteins found in flowering plants and seemed to be most similar to the rice RNMT (Supplementary Fig. S3C). Altogether, RLGS allowed the identification of DMRs encoding potential proteins with diverse biological functions and that were generated during vernalization and/or that distinguish sugar beet genotypes.

**DNA methylation profiles of DMRs**

DNA methylation patterns for the five annotated RLGS markers were assessed by MS-PCR using *McrBC* which cuts methylated DNA (Fig. 1A). Variations of methylation following cold exposure and/or between genotypes (S1 and R1 genotypes in Supplementary Fig. S4A at *JXB* online) were observed for each of them. A 3 week cold treatment was associated with the hypermethylation of *BvRTP*, *BvADT*, and *Bv4CL* or the hypomethylation of *BvRNMTa*, while *BvRNMTb* did not show clear variation. Differences between genotypes could also be observed for *BvRTP* and *BvRNMTa* without cold exposure. The five markers were hypomethylated after 18 weeks of cold exposure, except *BvRNMTb* in the S1 genotype (Supplementary Fig. S4A). Bisulphite sequencing revealed that the three cytosine contexts (CG, CHG, and asymmetric CCH, with H=A, T, or C) were methylated in *BvADT*, *BvRNMTa*, and *BvRNMTb* (Supplementary Fig. S5). Bioinformatic analyses revealed neither an miRNA target nor a TE in these five markers that could influence their methylation, except *BvRTP*. Cytosines exhibiting methylation polymorphism during vernalization and/or between genotypes belong to the three contexts and varied from 0 for overlapped spots (nos 4, 5, 13, and 17; Supplementary Table S2) to 30 out of 32 potential sites for marker 33 (data not shown). The variations of the methylation patterns during vernalization or between genotypes confirmed the MS-PCR profiles (Supplementary Fig. S5). For example, *BvADT* showed hypermethylation (13 sites out of 20) after 3 weeks of cold exposure in the S1 genotype. Altogether, RLGS markers exhibited variations of gene-body DNA hypo- or hypermethylation.

**Relative mRNA abundance of DMRs**

Variation in the relative mRNA abundance were observed for the five annotated RLGS markers during vernalization and/or between genotypes (Fig. 1B; Supplementary Fig. S4B at *JXB* online). The cold exposure induced an increase (BvRNMTb and Bv4CL) or decrease (BvRNMTa) in the mRNA abundance of the RLGS markers. Slight variations in relative mRNA abundance among genotypes were observed, such as for *BvRTP* (Fig. 1B). Two additional genotypes (S3 and R3) with intermediate bolting characteristics compared with S1 and R1 were analysed after 0, 3, and 18 weeks of cold exposure. The differential effect of a 3 week cold exposure was confirmed for *BvRNMTa* and *BvRNMTb*, but neither was expressed after 18 weeks of cold (Supplementary Fig. S4B). After cold exposure, the *Bv4CL* increase was confirmed in S3 but not in R3, which exhibited a decrease. These results showed that the five annotated protein-encoding DMRs exhibited distinct variations in their mRNA abundance (opposite for *BvRNMT a* and *b*) in the shoot apical meristem after cold exposure and/or between genotypes with distinct bolting tolerance.

**Role of DMRs in bolting**

In order to assess the potential role of sugar beet bolting DMRs, biological activities related to RNMT (RNA methylating) and ADT (phenolic compounds derived from phenylalanine) enzymes were measured in the shoot apical meristem of six genotypes with distinct bolting tolerance during 18 weeks of cold exposure (Fig. 2). The global RNA methylation level assessed by HPLC showed that S genotypes exhibited higher RNA methylation levels than R genotypes (Fig. 2A), with an absolute value of 2.4% before vernalization. In addition, a significant decrease in RNA methylation (21%) was observed only in S genotypes between 0 and 3 weeks of cold exposure (Fig. 2A). In order to test if the mRNA of vernalization genes such as *BvFT1*, *BvFT2* (Pin et al., 2011), and *BvFL1* (Reeves et al., 2007) could be targets of the RNA methylation pathway, MeRIP using 5-methylcytidine antibodies and semi-quantitative RT–PCR were performed on total RNAs from the shoot apical meristem of a resistant to bolting genotype (Fig. 2B). The specific expression patterns of these genes during vernalization were confirmed: *BvFT1* was expressed only before cold exposure, in contrast to *BvFT2*, while transcripts of *BvFL1* showed a slight increase at 4 °C. An amplification using immunoprecipitated RNA was only detected for *BvFL1* (two transcripts) after cold exposure. The housekeeping genes *BvTUBULIN* and *BvUBQUITIN* were expressed with or without cold treatment and were also methylated. These results suggest that RNA methylation could play a role in sugar beet bolting.

Analysis by HPLC of phenolic compounds derived from phenylalanine (synthesized by ADT) showed accumulation
in detectable amounts only after 3 weeks of cold exposure, whatever the genotype (Fig. 2C). Nine major HPLC peaks were identified (Fig. 2C) and their abundances were compared between S and R genotypes after 3 weeks at 4 °C (Fig. 2D). A significant difference was detected only for peak 4, showing a 4-fold increase in S genotypes (Fig. 2D). These data showed that cold exposure is associated with variations of secondary metabolism, with a slight difference between genotypes.

In order to characterize the bolting/flowering role of Arabidopsis homologues of RLGS sequences, lines carrying T-DNA insertions in homologues of the sugar beet RLGS sequences BvADT (lines SALK_041016 and SM_3.20773) or BvRNMTa and b (lines SALK_026873 and WiscDsLox508B11) were analysed. RT–PCR analyses revealed the highest decrease of ADT or RNMT expression in the adt (SALK_041016) and rnm (SALK_026873) mutants, respectively (Supplementary Fig. S2 at JXB online). HPLC analyses confirmed that both the RNA methylation level in the rnm mutant (Supplementary Fig. S2A) and the content of soluble phenolic compounds in the adt mutant (Supplementary Fig. S2) were lower than in the wild type, but not suppressed. Arabidopsis mutants were also studied for their bolting characteristics under three environmental conditions: short-day conditions with cold exposure (Fig. 3D, E) or without cold exposure (Fig. 3A–C), and long-day conditions without cold exposure (Supplementary Fig. S6). Bolting and flowering characteristics (BI, BD, FD, and the number of rosette leaves at bolting; see the Materials and methods) of rnm and adt mutants were significantly different from those of the wild type under all conditions. Under short days at 22 °C (non-vernalized), rnm plants exhibited an

![Fig. 1. DNA methylation and expression analyses of the five annotated RLGS markers during vernalization. (A) Methylation status by methyl-sensitive PCR (MS-PCR) analysis. Genomic DNA was extracted from the shoot apical meristem of one very sensitive very early bolting (S1) and one resistant late bolting (R1) sugar beet genotype exposed at 4 °C for 0 or 3 weeks. Genomic DNA was digested (+) or not (−) by the methyl-sensitive McrBC restriction enzyme (see the Materials and methods for details and controls) prior to PCR amplification, electrophoretic separation, and ethidium bromide staining. (B) mRNA relative abundance by semi-quantitative RT–PCR. Analyses were performed with reverse-transcribed total RNA isolated from the shoot apical meristem of one very sensitive very early bolting (S1) and one resistant late bolting (R1) sugar beet genotype exposed at 4 °C for 0 or 3 weeks. BvUBIQUITIN and BvTUBULIN were used as internal controls.](https://academic.oup.com/jxb/article-abstract/64/2/651/533218)
Fig. 2. (A) Global RNA methylation percentages. Total RNAs were extracted from shoot apical meristem of six vernalized sugar beet genotypes exposed at 4 °C during 0, 3, 12, 15, or 18 weeks, hydrolysed, and then separated by HPLC (see the Materials and methods). The global RNA methylation percentage was calculated as followed: \( \%MC = \frac{MC}{C+MC} \times 100 \) (S0
early and sensitive bolting (Fig. 3A, B) while adt showed a late and resistant bolting compared with the wild type (Fig. 3B). Indeed, after 14 weeks, non-vernalized rnm t plants reached 50% bolting (BD=68.9 d), whereas Col-0 plants reached only 11% (BD of 94.0 d) and adt plants did not bolt (Fig. 3B). Nevertheless, all the lines reached complete bolting after 20 weeks of culture. Two other mutant lines were studied under short days at 22 °C, rnm t‘ (line WiscDsLox508B11) and adt4 (line SM_3.20773), and the ranking of bolting tolerance observed between the three other Arabidopsis lines was confirmed (Supplementary Fig. S7). The number of rosette leaves at the time of bolting (Fig. 3C) and the bolting or flowering delays (Fig. 3C) were reduced in rnm t mutants and increased in adt mutants compared with Col-0. The two other lines, called adt4 (SM_3.20773) and rnm t‘ (WiscDsLox508B11), which displayed a weak decrease of the corresponding gene expression, were investigated under short days without cold exposure (Supplementary Figs S1, S7). These lines exhibited the same ranking of bolting tolerance as adt and rnm t compared with Col-0, but with smaller differences (Supplementary Fig. S7).

When plants under short days were vernalized (3 weeks of cold exposure at 4 °C), the ranking of bolting tolerance corresponds to sensitive genotypes before vernalization), where C is the amount of cytidine and MC is the amount of methylcytidine. The absolute %MC for S0 was 2.44%. Each bar corresponds to the mean of three genotypes resistant to (R, black bars) or three genotypes sensitive to bolting (S, white bars) with its corresponding standard error (n=9). Significant differences (at P ≤ 0.05) between R and S genotypic groups for each treatment duration are indicated by *, while significant differences between non-vernalized (0 week) and vernalized (3, 15, and 18 weeks) plants for a given genotypic group (R or S) are indicated by ″#″. (B) mRNA relative abundance by semi-quantitative RT–PCR for BvFT1, BvFT2, and BvFL1 (transcripts a and b from alternative splicing; see Reeves et al., 2007) during vernalization in total RNA and methylated RNA obtained by immunoprecipitation with 5-methylcytidine antibodies (see details in the Materials and methods). Analyses were performed with reverse-transcribed total RNA isolated from the shoot apical meristem of sugar beet exposed at 4 °C for 0 or 9 weeks. The number of PCR cycles was adapted using BvUBIQUITIN and BvTUBULIN (internal controls) to obtain similar amplification for these housekeeping genes between immunoprecipitated and total RNAs. (C) Example of HPLC chromatograms obtained for soluble phenolic compounds extracted from the shoot apical meristem of one very resistant, very late bolting sugar beet genotype (R4) after 0, 3, or 18 weeks at 4 °C. For clarity of the results, data obtained after 12 or 15 weeks at 4 °C are not shown. The nine major HPLC peaks absorbing at 280 nm (numbered from 1 to 9) are indicated by arrows. (D) Relative amounts for the nine major HPLC peaks in three genotypes resistant to (black bars) or three genotypes sensitive (white bars) to bolting after 3 weeks at 4 °C. Each bar corresponds to the mean with its corresponding standard error (n=9). Significant differences (at P ≤ 0.05) between genotypes resistant and sensitive to bolting are indicated by *.

Fig. 3. Bolting and flowering characteristics of vernalized (D and E) and non-vernalized (A to C) Arabidopsis in short-day conditions for wild-type (Col-0), rnm t (RNA METHYLCYTOSINE TRANSFERASE T-DNA insertion line), and adt (AROGENATE DEHYDRATASE T-DNA insertion line) accessions. (A) Phenotype of 14-week-old non-vernalized plants of Col-0 and rnm t lines. (B and D) Kinetics of the bolting index (BI) corresponding to the
between the three Arabidopsis lines was conserved, but with increased differences between them (Fig. 3D). Vernalization was shown to accelerate bolting and flowering in Col-0 and rmnt lines, but not in adt plants (Fig. 3D, E). Indeed, after 14 weeks, vernalized rmnt plants reached 100% bolting (BD of 62.7 d), while Col-0 plants reached 46% bolting (BD of 72.2 d) and adt plants did not bolt (Fig. 3D). Nevertheless, all the lines reached complete bolting after 20 weeks of culture. The number of rosette leaves, and the bolting and flowering delays were lower in rmnt vernalized plants and higher in adt plants than in controls (Fig. 3E). Interestingly, the kinetics of BIs during development of non-vernalized rmnt plants was similar to those of vernalized Col-0 plants (Fig. 3B, D).

Under long-day conditions without cold exposure, bolting and flowering were strongly accelerated (BI=100% before 10 weeks) compared with short-day conditions (Supplementary Fig. S6). A conserved ranking of bolting tolerance between the three Arabidopsis lines could be observed. Indeed, after 6 weeks, vernalized rmnt plants had 86% bolting (BD of 37.6 d) while Col-0 plants showed 81% bolting (BD of 38.0 d) and adt plants displayed 47% bolting (BD of 39.5 d) (Supplementary Fig. S6A at JXB online). The number of rosette leaves at the time of bolting was reduced significantly in rmnt plants and bolting/flowering delays were significantly increased in adt plants compared with Col-0 (Supplementary Fig. S6B, C).

Overall, these observations showed that both rmnt and adt play a role in the bolting/flowering transition in Arabidopsis.

Discussion

DNA methylation generates gene-body DMRs during vernalization in sugar beet genotypes

The useful application of high-throughput methodologies for genome-wide analysis is dependent on the availability of genomic resources and is currently limited for many crop plants such as sugar beet (Miguel and Marum, 2011). In such cases, RLGS is an interesting alternative providing a direct, rapid, and low-cost quantitative epigenetic assessment, in several biological conditions at the same time, of several CG methylated genes without prior knowledge of gene sequence (Costello et al., 2002). Several publications demonstrate the successful use of RLGS in plants (Causevic et al., 2006; Takamiya et al., 2008; Okuizumi et al., 2011; Maury et al., 2012).

In the present study, 39 DMRs were identified, ranging from 121 bp to 859 bp, in agreement with a previous study (Causevic et al., 2006; Maury et al., 2012). Furthermore, DMRs were generated according to the duration of cold exposure (33 RLGS markers out of 39) and/or between genotypes (14 RLGS markers out of 39), in agreement with the reported variations of global DNA methylation (Trap-Gentil et al., 2011). Moreover, the majority of the RLGS markers had potential ORFs, suggesting that gene-body DNA methylation on CG sites in sugar beet is affected by both environmental conditions and genetic background. Several recent studies have mapped the distribution of cytosine methylation in the entire genome (i.e. methylome) of Arabidopsis (Zhang et al., 2006; Vaughn et al., 2007; Zilberman et al., 2007; Cokus et al., 2008; Lister et al., 2008) and showed that gene-body regions are targeted for cytosine methylation. Almost all of the RLGS markers contained CpG islands, as expected from the use of the NorI enzyme with a GC↓GGCCGC recognition sequence. The role and existence of CpG islands in plants are still not clear and seem to vary between species (Masoudi-nejad et al., 2011).

Bolting DMRs display genotype-dependent DNA hyper- or hypomethylation after cold exposure that may be conserved between genotypes with similar bolting tolerance

For all RLGS markers tested, MS-PCR and bisulphite sequencing confirmed DNA methylation variations during vernalization and/or between genotypes, strengthening the relevance of RLGS in crop plants (Maury et al., 2012). Markers exhibited DNA hypo- (BvRNMTa) or hypermethylation (BvRTP, BvADT, and Bv4CL) after a 3 week exposure to cold. Global DNA hypermethylation has already been reported in sugar beet after short durations of cold exposure (Trap-Gentil et al., 2011) as well as for BvCACTA (CACTA transposon family) and Bv5S (SS rRNA) genes. Interestingly, BvRNMTa exhibited DNA hypomethylation after a 3 week cold exposure, as previously shown for the two vernalization genes BvFL1 and BvVIN3 (Trap-Gentil et al., 2011). After a long cold exposure (18 weeks), hypomethylation of the markers was observed, in agreement with the global DNA hypomethylation previously reported in the shoot apex (Trap-Gentil et al., 2011). The kinetics of DNA methylation could indicate successive determination steps of the shoot apical meristem state (with activation/repression and hyper-/hypomethylation of various loci) leading to bolting. These data showed that the DNA methylation machinery could specifically target genes in response to developmental or environmental signals in plants (Zhang et al., 2010; Maury et al., 2012) and particularly during vernalization (Trap-Gentil et al., 2011; Vanyushin and Ashapkin, 2011). RLGS markers (BvRTP and BvRNMTa) were also shown to display DNA hypo- or hypermethylation between genotypes. This is in agreement with our previous report (Trap-Gentil et al., 2011) which showed that bolting-sensitive genotypes are more methylated than resistant ones throughout vernalization and that the methylation profiles of BvFL1 and BvVIN3 varied among sugar beet genotypes.
DMRs encode potential enzymes and exhibit variations of their expression during vernalization

In the present study, a functional annotation was only found for five RLGS markers: a putative retrotransposon (BvRTP) and four enzymes: an AROGENATE/PREPHRENATE DEHYDRATASE (BvADT) and a COUMARATE: COENZYME A-LIGASE (Bv4CL), both involved in phenylalanine metabolism, and two RNA METHYLICYTOSINE TRANSFERASES (BvRNMT a and b). Cytosine methylation of TE s has been proposed to protect genomes against endogenous 'selfish' DNA (Zilberman, 2008). BvRTP displayed hypermethylation and a decrease in expression after a 3 week cold exposure but only in the sensitive genotype. BLASTN analyses revealed that this retrotransposon was situated adjacent to an ARGinine DECARBOXYLASE (ADC; data not shown), required for the biosynthesis of putrescine which controls cell division and responses to biotic and abiotic stresses (Kumar et al., 1997). In rice, retrotransposons were found to be able to regulate the expression of adjacent genes in their host genomes (Kashkush and Khasdan, 2007). Further analyses on BvRTP will be necessary to clarify this point.

The last four DMRs encoding enzymes were all shown to display changes in mRNA accumulation after cold exposure and/or between genotypes. Accordingly, changes in the epiallelic state have recently been shown to lead to major effects on transcriptional output in Arabidopsis (Schmitz et al., 2011). Interestingly, BvRNMT a and b, exhibiting distinct gene-body DNA methylation profiles, were shown to be activated (BvRNMTb) or silenced (BvRNMTa) by a 3 week exposure to cold. Vernalization depends on intricate epigenetic regulation at the transcriptional level during plant development (Zhang et al., 2010). The biological significance of gene-body methylation is still not clear, although it has been proposed that it may suppress aberrant transcription from cryptic promoters inside the genes (Zilberman et al., 2007; Lauria and Rossi, 2011). Emerging lines of evidence hint that the roles of cytosine methylation are diverse and probably individualized for different genes (Zhang et al., 2010).

Role of AROGENATE DEHYDRATASE and phenylpropanoid metabolism in bolting

The markers 18 and 37 encode putative enzymes both involved in the metabolism of phenylalanine. In plants, phenylalanine serves as a building block for proteins and a wide range of aromatic compounds (Cho et al., 2007), such as phenolic compounds. After 3 weeks of cold exposure, a transient and strong relative accumulation of soluble phenolic compounds was found in sugar beet shoot apical meristems. Interestingly, the abundance of one compound (no. 4) was 4-fold less in cold-treated R genotypes compared with cold-treated S genotypes, in agreement with the decrease of BvADT mRNA abundance in cold-treated R genotypes. This phenolic compound has already been described in sugar beet cell lines by liquid chromatography–mass spectrometry (m/z ratio of 576; Causevic et al., 2006) and could correspond to tannin (Bystrom et al., 2008). The adt Arabidopsis mutant displayed a significantly lower amount of soluble phenolics than the wild type. No comparison could be made with sugar beet phenolic composition which was different. Recently, the zce1 (Zusammen cis-Cinnamic acid Enhanced) loss-of-function mutant produced an earlier bolting phenotype in Arabidopsis, suggesting that ZCE1, whose expression is activated by cis-cinnamic acid, plays a role in promoting vegetative growth and delaying flowering (Guo et al., 2011).

In Arabidopsis, ADT belongs to a multigene family encoding six ADTS (ADT1–ADT6). The mutant line used in the present study has a T-DNA inserted in the ADT1 gene, which is widely expressed in Arabidopsis (Supplementary Fig. S8A at JXB online) and methylated in the CG context (Supplementary Fig. S8B). Bolting and flowering characteristics of this mutant were significantly affected compared with the wild type, the adt mutant exhibiting a resistant late bolting/flowering phenotype. This is in agreement with the decreased level of ADT expression after cold treatment observed in the resistant sugar beet genotype. In addition, under short days, the cold exposure did not accelerate bolting and flowering of adt plants. These data suggest that ADT contributes to the timing of bolting and flowering, particularly in response to vernalization, possibly through the production of phenylpropanoid compounds; further studies will be necessary to clarify its role.

Role of two RNA METHYLTRANSFERASE genes and RNA methylation in bolting: control of vernalization at the RNA level

In the present report, two sequences encoding similar proteins, RNA METHYLICYTOSINE TRANSFERASES (BvRNMTa and b), were identified as DMRs for bolting. Interestingly, BvRNMTa displayed gene-body hypomethylation and silencing of expression after cold exposure, while BvRNMTb was hypermethylated (bisulphite sequencing) and activated by a 3 week cold exposure, with slight differences between genotypes. A similar antagonistic situation has already been reported for the two BvFT1 and BvFT2 sugar beet paralogues controlling bolting induction. However, BvFT1 and BvFT2 showed 82% protein identity (Pin et al., 2010), while the two BvRNMTs shared 97% identity (in a conserved protein domain, S-adenosylmethionine-dependent methyltransferase superfamily domain). Recently, alleles of the sugar beet Bolting locus were shown to differ by only 11 non-synonymous single nucleotide polymorphisms, and the same haplotype (Bvbtc1) was found in all cultivated accessions (Pin et al., 2012). This rare recessive allele Bvbtc1 in biennials cannot transduce the sensitivity to photoperiod (in contrast to BvBTC1 in annuals) but retained a role as a promoter of bolting under vernalization. Altogether, the present data report the antagonistic patterns of two BvRNMT sequences, but further analyses, with the availability of the sugar beet genome information, will clarify their genic versus allelic status and will help to understand the potential significance of their antagonistic behaviours on RNA methylation (distinct mRNA targets or distinct levels of activity).
The global RNA methylation level in sugar beet shoot apex was very low (≈2.4%), in agreement with other studies (Dubin and Taylor, 1975; Squires et al., 2012), and decreased early during cold exposure only in the bolting-sensitive genotype. The expression profiles of the BvFT1, BvFT2, and BvFL1 vernalization genes (Reeves et al., 2007; Pin et al., 2010; Trap-Gentil et al., 2011) were assessed, as well as their methylation state using a new approach: MeRIP. This analysis revealed the methylation and expression of BvFL1 mRNA only, in shoot apical meristem of a bolting-resistant genotype after cold exposure. This is in agreement with existing reports of BvFL1 expression in vernalized shoot apex (Reeves et al., 2007; Trap-Gentil et al., 2011) and with its repressing effect on flowering (Reeves et al., 2007). BvTUBULIN and BvUBIQUITIN mRNA were found to be methylated in all conditions, confirming the observations of Squires et al. (2012). Methylation of mRNA was detected many years ago, but its role is still unclear and could promote efficient mRNA translation (Squires and Preiss, 2010, 2011). Conventional bisulphite sequencing (Schafer et al., 2008) failed to give significant results on sugar beet mRNAs (data not shown), certainly due to their very low methylation level. Next-generation RNA-Seq following bisulphite treatment, as reported in Squires et al. (2012), should be better adapted to the identification of methylation in mRNA.

An Arabidopsis rnmtn mutant that displayed lower levels of RNA methylation than the wild type was studied. This T-DNA line is mutated in the RNMT gene (At3g13180), which belongs to a multigenic family (Pavlopoulou and Kossida, 2009) and is widely expressed in Arabidopsis, with raised expression in the shoot apex and vegetative rosette (Supplementary Fig. S9A at JXB online). RNMT is methylated in CG contexts in Arabidopsis, and the corresponding mRNA accumulation is slightly increased in the DNA methyltransferase Arabidopsis mutants (Supplementary Fig. S9B). Furthermore, RNMT expression is strongly decreased in Arabidopsis after the flowering transition and is slightly increased in lines which are mutated for met1 or for the vip5 and vip6 (VERNALIZATION INDEPENDENCE) genes (Oh et al., 2004). These genes repress flowering by stimulating FLC expression. Bolting and flowering of this mutant were significantly affected compared with the wild type, the rnmtn mutant exhibiting an early and sensitive bolting/flowering phenotype. This is consistent with the early decreased level of RNA methylation after cold treatment which was observed in the sensitive sugar beet genotypes. In addition, under short days, exposure to cold still accelerates bolting and flowering of rnmtn plants. Interestingly, the bolting kinetics of rnmtn plants without cold exposure were similar to those of vernalized control plants. A similar situation has already been described for the met1 DNA methyltransferase-deficient plants (Finnegan et al., 1998). Together, these data suggest that RNMT participates in the timing of bolting and flowering, but seems not to be necessary for the formation of the bolted stem and flowers.

The complete genomic sequence of sugar beet as well as quantitative trait loci for bolting tolerance are not yet available and will be important to develop sugar beet breeding. Nevertheless, RNMT seems to represent an interesting candidate for further studies on bolting. In addition, a recent study using RNA-seq to establish a sugar beet transcriptome identified other candidate genes for vernalization and showed that transcripts encoding a BvRAV1-like (Related to ABA-insensitive 3/viviparous1) protein are strongly up-regulated by vernalization, suggesting a hitherto unsuspected role for this protein family in the vernalization response (Mutasa-Gottgens et al., 2012). Thus next-generation sequencing, in association with powerful bioinformatic tools, will allow the genome-wide identification of new candidate genes for bolting.

Supplementary data
Supplementary data are available at JXB online.

Figure S1. mRNA relative abundance, global RNA methylation percentages, and soluble phenolic compounds in Arabidopsis accessions (Col-0, rnmtn, rnmtn’, adt, and adt4).

Figure S2. Longitudinal section of a 26-week-old sugar beet shoot apex and scanning of CpG islands methylation by restriction landmark genome scanning (RLGS).

Figure S3. Sequence analyses of BvRNMTa (23) and BvRNMTb (32).

Figure S4. DNA methylation and expression patterns for the five annotated RLGS markers during vernalization.

Figure S5. Bisulphite data for BvADT (18), BvRNMTa (23), and BvRNMTb (32) during vernalization.

Figure S6. Kinetics of the bolting index (BI) of the non-vernalized Arabidopsis accessions (Col-0, rnmtn’, and adt4) in short-day conditions.

Figure S7. Bolting and flowering characteristics of non-vernalized Arabidopsis accessions in long-day conditions (Col-0, rnmtn, and adt).

Figure S8. Microarray data on AtADT1 expression and methylation in Arabidopsis public databases.

Figure S9. Microarray data on AtRNMT expression and methylation in Arabidopsis public databases.

Table S1. Sequences of primers used in methyl-sensitive PCR, semi-quantitative RT–PCR, and bisulphite sequencing experiments.

Table S2. Characteristics of the 39 sugar beet genomic sequences isolated by restriction landmark genome scanning (RLGS markers).

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RNA methyltransferase, arogenate dehydratase and bolting


