A Century-Old Mystery Unveiled: Sekizaisou is a Natural Lignin Mutant[1][OPEN]

Dear Editor,

Lignin is a major polymer in the vascular plant secondary-thickened cell wall. This aromatic polymer provides hydrophobicity and strength to the cell wall, thereby facilitating vascular conductivity and upward growth. Mutations in lignin biosynthetic genes induce qualitative and/or quantitative alterations of the polymer (Boerjan et al., 2003). Such mutations are sometimes accompanied by changes in the color of the xylem cell walls. Although the color change is an indicator of lignin alteration, assessing color differences is extremely difficult based on only external appearance, especially in woody plant species, because xylem is hidden by bark. This may be one of the reasons why the discovery of naturally occurring lignin mutants in trees is quite rare (Ralph et al., 1997; Marroni et al., 2011; Vanholme et al., 2013). Here, we report a naturally occurring mulberry (*Morus alba*), 'Sekizaisou', which is deficient in a gene encoding CINNAMYL ALCOHOL DEHYDROGENASE (CAD), which catalyzes the last step in lignin monomer biosynthesis.

Mulberry trees (*Morus* sp.) are grown widely for their fruit and silk production. Their leaves contain all the essential chemical components for silkworm (*Bombyx mori*) growth and development. According to Yoshimura and Saito (1924), a mulberry tree with drooping branches (Supplemental Fig. S1), reminiscent of those in weeping willow, and unusual red-colored wood was discovered around 1912 by a farmer in bushland on Okushiri Island, northern Japan. It was locally called "Kusuri-guwa (medicine mulberry)" or "Murasaki-guwa (purple mulberry)" a century ago and was believed by people on the island to be a medicinal herb (Hotta, 1937). In 1950, the striking appearance of this tree's red-colored wood was immortalized in a painting published in a book written by Hotta (1950; Fig. 1A). Grafted plantlets of cv Sekizaisou were established in Tokyo in 1922, and the propagules have been preserved by sequential vegetative propagation ever since (Supplemental Materials and Methods). The tree was named cv Sekizaisou ("Sekizai" means "red colored wood" and "sou" means "mulberry" in Japanese) by Yoshimura and Saito (1924). The leaves of this tree were used as feed for sericulture on Okushiri Island from 1916 until around the 1930s, because of their positive effects on silkworm growth and silk cocoon quality compared with those from other local mulberry cultivars planted on the island (Yoshimura and Saito, 1924).

F1 progenies derived from crosses between cvs Sekizaisou (seed parent) and Kokusou 21 (pollen parent) had neither drooping stems nor red-colored xylem (Supplemental Fig. S2). This suggested that a homozygous recessive gene caused the morphological characteristics and the red-colored wood. The characteristics that typify cv Sekizaisou resemble those of mutants and transgenic plants with altered lignin structure (Fig. 1; Supplemental Fig. S2).

Lignin is derived from the polymerization of the monolignols *p*-coumaryl, coniferyl, and sinapyl alcohols, called monolignols, producing *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units in the polymer (Fig. 2), respectively (Boerjan et al., 2003; Vanholme et al., 2019). The last step in monolignol biosynthesis, the reduction of an aldehyde to an alcohol, is catalyzed by CAD (Fig. 3). Plants with reduced CAD activity typically have a brown- or red-colored xylem (Baucher et al., 1996; MacKay et al., 1997; Pilate et al., 2002), and have often insufficient strength to allow the plants to stand fully upright (Sibout et al., 2005). These observations suggested that cv Sekizaisou may be a natural mutant with altered lignin structure due a mutation in *CAD*.

We evaluated the lignin amount and composition of cv Sekizaisou along with six other cultivars that served as controls (Supplemental Table S1). The lignin content of the extractive-free wood was determined via the acetyl bromide protocol and appeared significantly lower in cv Sekizaisou (14.9% [w/w]) than in the other examined cultivars (20.1% to 22.3%; Supplemental Table S1). The lignin monomeric composition was determined by the cleavage of the ether linkages via thioacidolysis, followed by the detection of the released

[1]This work was supported by the Japan Society for the Promotion of Science (JSPS) (JP18K45678 to S.K.), Japan Science and Technology Agency (JST) (JPMJAL1107 to N.M.), and DOE | Office of Under Secretary for Science (DE-SC0018409).

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M.Y. analyzed lignin structure by thioacidolysis; M.Y. and N. prepared milled wood samples; H.T., A.K., and H.O. propagated cv Sekizaisou vegetatively and generated its F1 progenies; H.T., A.K., H.O., W.B., J.R., and S.K. designed the study; H.T., A.K., Y.H., and H.S. prepared the samples for metabolomic analysis; S.L., H.K., and J.R. prepared samples for NMR and performed NMR analysis and interpretation of the data; R.V. and G.G. analyzed the metabolites in different mulberry cultivars and interpreted the data; R.V., H.K., W.B., J.R., and S.K. improved the manuscript; Y.H. and S.K. analyzed the genomes of cvs Sekizaisou and Nezumigaeshi; N. and M.U. managed cultivated plants; N.T. and T.I. contributed to histochemical analysis and pulp preparation, respectively; S.S. and N.M. analyzed the monomeric composition of cell wall polysaccharides; N.M. and S.K. managed the research project; S.K. wrote the initial draft of the manuscript; all authors approved the final version of the manuscript.

[OPEN]Articles can be viewed without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.19.01467

Figure 1. Morphological characteristics of cv Sekizaisou. A, A hand-made painting of debarked branching stems of cv Sekizaisou (left) and a control mulberry (right) published by Hotta (1950). B and C, cv Sekizaisou plantlets growing in the experimental field in early summer and midsummer, respectively. The creeping growth habit can be observed for the branches originating from the bottom part of the stumps (white arrows in B). Unlike other cultivars growing upright, such as the plants growing behind cv Sekizaisou, most of the branches of cv Sekizaisou drooped (white arrows in C), and supporting poles were necessary to maintain upward growth of the main stems. D, Debarked branches of cv Sekizaisou (left) and cv Nezumigaeshi (right) prepared from plants in midsummer. E, Longitudinal sections of top (right) and bottom (left) parts of branches of cv Sekizaisou. The red color of the inner xylem, close to the pith parenchyma, was lighter than that of the outer xylem. X and P indicate xylem and parenchyma tissues, respectively. F, Debarked stems of cv Sekizaisou (left) and cv Nezumigaeshi (right) after air-drying for 3 d. The xylem tissue of cv Sekizaisou turned brown. G, Wood powder of extractive-free wood from cv Sekizaisou (left) and cv Nezumigaeshi (right). The resultant brown pigment in air-dried xylem could not be removed from the powder upon sequential extraction with hot water and organic solvents.

monomers via gas chromatography (GC)–flame-ionization detection (FID). Conventional G and S thioacidolysis-released monomers ($\mathbf{2}_G$ and $\mathbf{2}_S$, respectively) were detected in all samples. The S/G ratio was lower in cv Sekizaisou (0.78) than in the other examined cultivars (0.99–1.19; Supplemental Table S1), indicative of the fact that lignin biosynthesis is altered in cv Sekizaisou.

A deficiency in CAD activity typically induces the substantial incorporation of its substrates, coniferaldehyde, and sinapaldehyde, into the lignin polymer, giving rise to various substituted coniferaldehyde (G′) and sinapaldehyde (S′) units in the lignin (Fig. 2). Thioacidolysis-released indenes ($\mathbf{1}_G$ and $\mathbf{1}_S$; Kim et al., 2002; Lapierre et al., 2004), which are indicative of the incorporation of coniferaldehyde and sinapaldehyde into the lignin, may be detected via GC-FID, but GC-mass spectrometry (MS) is required for proper authentication (Supplemental Materials and Methods). Therefore, thioacidolysis products of wood from cv Sekizaisou were analyzed via GC-MS and compared with those of cv Nezumigaeshi used as a control mulberry cultivar, and to those of a transgenic CAD-deficient poplar (*Populus tremula* × *Populus alba*) used as a positive control for the incorporation of *p*-hydroxycinnamaldehydes into the lignin (line hpCAD19; Van Acker et al., 2017). The thioacidolysis-released indenes $\mathbf{1}_G$ (*m/z* 293 and 354) and $\mathbf{1}_S$ (*m/z* 293, 323 and 384), were observed in samples from 'Sekizaisou' and the CAD-deficient poplar (denoted as $\mathbf{1}_{G1}$, $\mathbf{1}_{S1}$, $\mathbf{1}_{S2}$, and with an asterisk

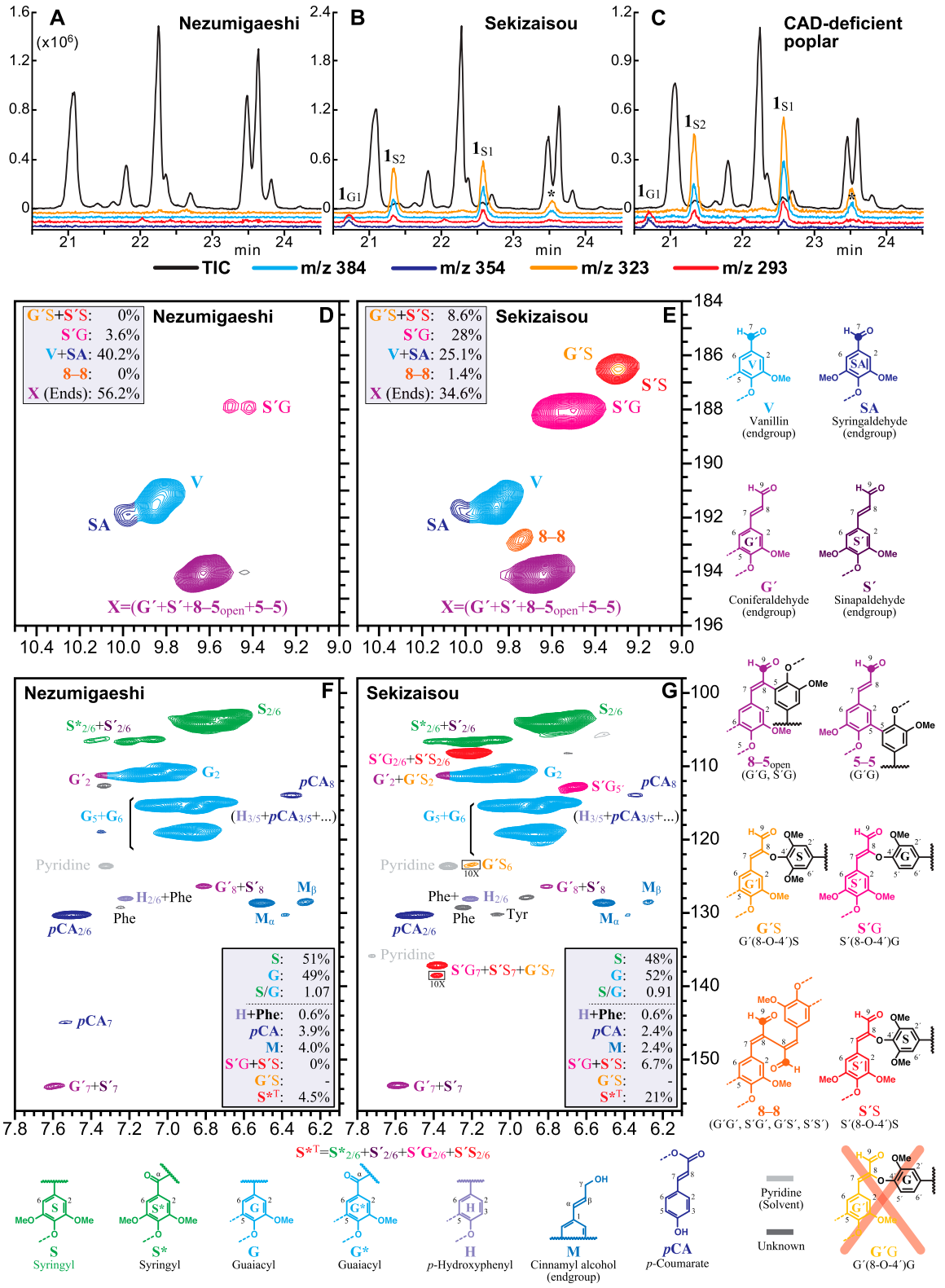

Figure 2. GC-MS and HSQC analysis. Partial GC-MS traces showing the detection of the diagnostic indene markers released by thioacidolysis of lignins in cv Nezumigaeshi (A), cv Sekizaisou (B), and CAD-deficient transgenic poplar (C). Peaks labeled $\mathbf{1}_{G1}$, $\mathbf{1}_{S2}$, and $\mathbf{1}_{S1}$ correspond to the indenes whose structures are shown in Supplemental Figures S3 and S4. Total-ion (TIC) and selected-ion (*m/z* 293, 323, 354, and 384) chromatograms are indicated with different colors. The selected-ion chromatograms are shown at 100-fold magnification, and the *y* axis zero-point is offset so that the chromatograms are more clearly resolved and viewed. Representative chromatograms are

in Fig. 2, B and C), but not in those from 'Nezumigaeshi' (Fig. 2A; for mass spectra and structures of the indenes see Supplemental Figs. S3 and S4). Although the peak eluting at 23.5 min, and which is indicated with an asterisk in Figure 2, B and C, could not be identified, these data indicate that the lignin from cv Sekizaisou contains increased levels of sinapaldehyde and, to a lesser extent, also coniferaldehyde, as observed in other CAD-deficient plants (Kim et al., 2002; Lapierre et al., 2004; Van Acker et al., 2017).

To further elucidate the nature of the lignin, ^{1}H–^{13}C heteronuclear single-quantum correlation spectroscopy (HSQC) was performed on the lignin isolated from cv Sekizaisou and from the control cv Nezumigaeshi (Fig. 2, D–G). The aldehyde spectral region (Fig. 2, D and E) is the most diagnostic feature for the incorporation of the various hydroxycinnamaldehydes. As in the lignin of other angiosperms, a peak corresponding to **S′**(8–O–4)G structures, derived from 8–O–4 cross-coupled sinapaldehyde in the polymer, could be detected at a low level in cv Nezumigaeshi (Fig. 2D). Contrarily, in cv Sekizaisou (Fig. 2E), it was evident that coniferaldehyde and sinapaldehyde were integrally coupled at much higher levels into the lignin polymer via 8-coupling into **G′**(8–O–4)S (from coniferaldehyde), and **S′**(8–O–4)G and **S′**(8–O–4)S (from sinapaldehyde) structures. As the coniferaldehyde was found to be unable to couple with G units in previous studies, such as in synthetic lignin oligomers (Kim et al., 2000) and CAD-deficient tobacco (*Nicotiana tabacum*; Kim et al., 2003), and later reported in CAD-deficient poplar (Van Acker et al., 2017), we assumed that there are no **G′**(8–O–4)G-structures in the lignin of cv Sekizaisou. A cross-peak from homodimerization via 8–8-coupling was also detected, indicating that a sufficient number of aldehyde monomers was exported into the cell wall that their homodimerization could initiate polymerization, as also reported in CAD-deficient tobacco (Kim et al., 2003) and poplar (Van Acker et al., 2017).

To examine the effect of the putative mutation in *CAD* on soluble phenolics, including low M_r lignin precursors, methanol extracts from stems of field-cultivated mulberry plants were analyzed by using UHPLC/MS (Fig. 3; Supplemental Fig. S6; Supplemental Tables S2, S3, and S4). As no near-isogenic lines of cv Sekizaisou are available, the methanol-soluble phenolics from a single plant of each of 51 different mulberry cultivars belonging to *M. alba* were profiled as controls. A list of 208 compounds (166 more and 42 less abundant) with significantly different signal intensities between the control (average for 51 cultivars) and Sekizaisou was obtained; of these, 21 compounds (16 more and 5 less abundant) could be structurally characterized.

Most of the more abundant metabolites that could be structurally characterized were phenylpropanoids from the canonical monolignol pathway (compounds **1** and **13**; Fig. 3; Supplemental Table 2) and from pathways branching therefrom. As in a previous study of the transgenic poplar with suppressed CAD activity (Van Acker et al., 2017), the abundance of syringyl lactic acid hexoside (compound **12**) and an oxidatively coupled homodimer of sinapaldehyde, S′(8–8)S′ (compound **14**) were significantly higher in 'Sekizaisou' compared with the controls. In addition, a dimer (compound **15**) and a trimer (compound **16**) derived from sinapaldehyde were also more abundant in cv Sekizaisou. By contrast, a trimer and a tetramer from the canonical monolignols, in addition to two such dimer hexosides, were less abundant in cv Sekizaisou. The aromatic amino acid, Trp, was also lower in abundance in cv Sekizaisou. A lower abundance of Trp has not been reported before in other lignin mutants. Collectively, these data strongly suggested that cv Sekizaisou is indeed a *CAD*-deficient mulberry plant.

As reported by Yoshimura and Saito (1924), the leaves of cv Sekizaisou had been used as feed for silkworms for local sericulture on Okushiri Island. Caffeoyl quinic acid (chlorogenic acid), for which the biosynthetic pathway overlaps with that of monolignols, is known as a growth-promoting factor for the silkworm (Kato and Yamada, 1964). Caffeoyl quinic acid (compound **2**) also significantly accumulated in stems of cv Sekizaisou compared with the control (Fig. 3; Supplemental Table S2). Future studies will determine whether leaves from cv Sekizaisou have a positive effect on the feeding quality compared with its controls.

To investigate whether the observed morphological and molecular phenotypes of cv Sekizaisou were truly caused by a deficiency in *CAD*, the genomes of cv Sekizaisou and the control cv Nezumigaeshi were sequenced by using the HiSeq X Ten (Illumina) platform (Supplemental Materials and Methods). By using a BLAST search, we found three putative *CAD* gene sequences in the draft genome of cv Sekizaisou (gene identifcations, 2117-RA, 2718-RA, and 9780-RA). Of these, 9780-RA was the closest ortholog to *MnCAD1* (98.6% coding sequence identity), the *CAD* gene that was predicted to be involved in lignin biosynthesis in *Morus notabilis* (Fig. 4B; Supplemental Fig. S5; He et al., 2013). We therefore designated 9780-RA as *Sekizai-CAD1*. The coding regions of the other two *CAD* homologs, 2117-RA and 2718-RA, exhibited only 56.5% coding sequence identity with *MnCAD1*, respectively. To further

Figure 2. (*Continued.*)
shown for 3 independent biological samples. D to G, Two-dimensional heteronuclear single-quantum coherence NMR spectra of cellulolytic enzyme lignins from cv Nezumigaeshi (D and F) and cv Sekizaisou (E and G). The spectra show the aldehyde (D and E) and aromatic (F and G) structures in both lignins. The quantification values shown are for relative comparisons of the lignin components determined from NMR contour volume-integrals. Correlation peaks are colored to match those of the structures; Phe is from Phe (in proteins), and Tyr is from Tyr (in proteins); the $H_{2/6}$ correlation peak overlaps with a Phe peak so cannot be properly quantified.

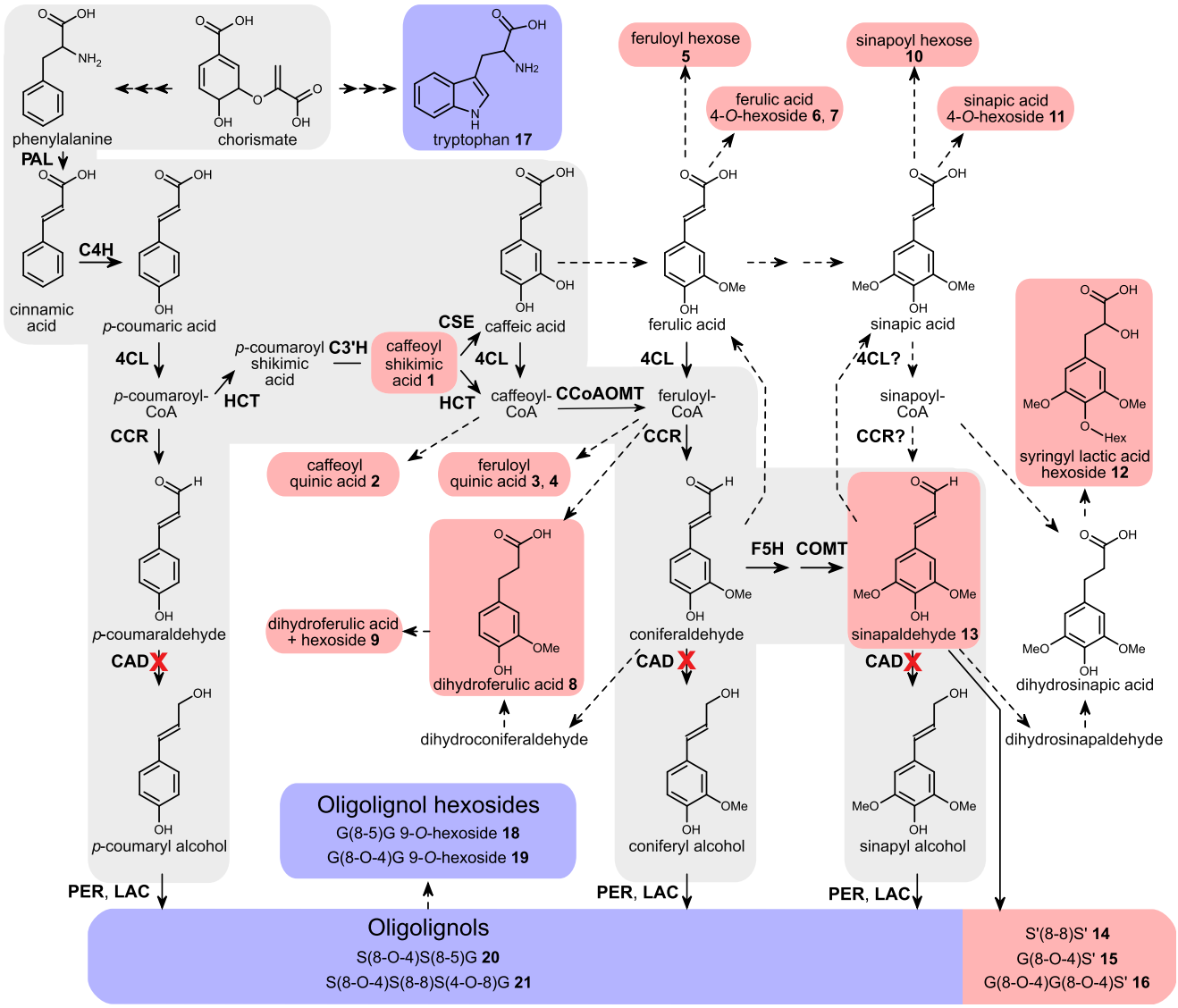

Figure 3. Metabolic pathway based on phenolic profiling. Methanol-soluble phenolics differentially accumulated between Sekizaisou and other cultivars. The molecular structures of the individual metabolites can be found in Supplemental Figure S6 and Supplemental Table S2. The canonical monolignol biosynthetic pathway is shown with a gray background. As no near-isogenic line of cv Sekizaisou can be provided at present, the methanol-soluble phenolics from a single plant of each of 51 different mulberry cultivars (Supplemental Tables S3) that had been cultivated in the same experimental field and belonged to *M. alba* were profiled as controls. More and less abundant metabolites in cv Sekizaisou compared with the other cultivars are represented with red and blue backgrounds, respectively. The dashed arrows indicate one or more proposed conversions. C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaric acid:CoA ligase; C3′H, *p*-coumaroyl quinate/shikimate 3′-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamoyl-CoA reductase; COMT, caffeate *O*-methyltransferase; CSE, caffeoyl shikimate esterase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; F5H, ferulate 5-hydroxylase; HCT, hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyl transferase; LAC, laccase; PAL, Phe ammonia lyase; PER, peroxidase.

confirm the nucleotide sequence of 9780-RA, we performed PCR of this region, followed by sequencing of the amplicon (Supplemental Table S5).

Based on the sequencing results, the *Sekizai-CAD1* locus in cv Sekizaisou is homozygous (accession no., LC476972) and the *CAD1* locus in cv Nezumigaeshi is heterozygous (LC476973 and LC476974). When the sequence of *Sekizai-CAD1* was compared with the *MnCAD1* and *CAD1* sequences from cv Nezumigaeshi, a guanine insertion was detected close to the predicted translation start codon of *Sekizai-CAD1* (Fig. 4; Supplemental Fig. S5). This mutation was also confirmed by direct sequencing of the first-strand cDNA derived from the reverse transcripts of *Sekizai-CAD1* mRNA. This insertion caused a frameshift and, consequently, a premature stop codon in the putative first

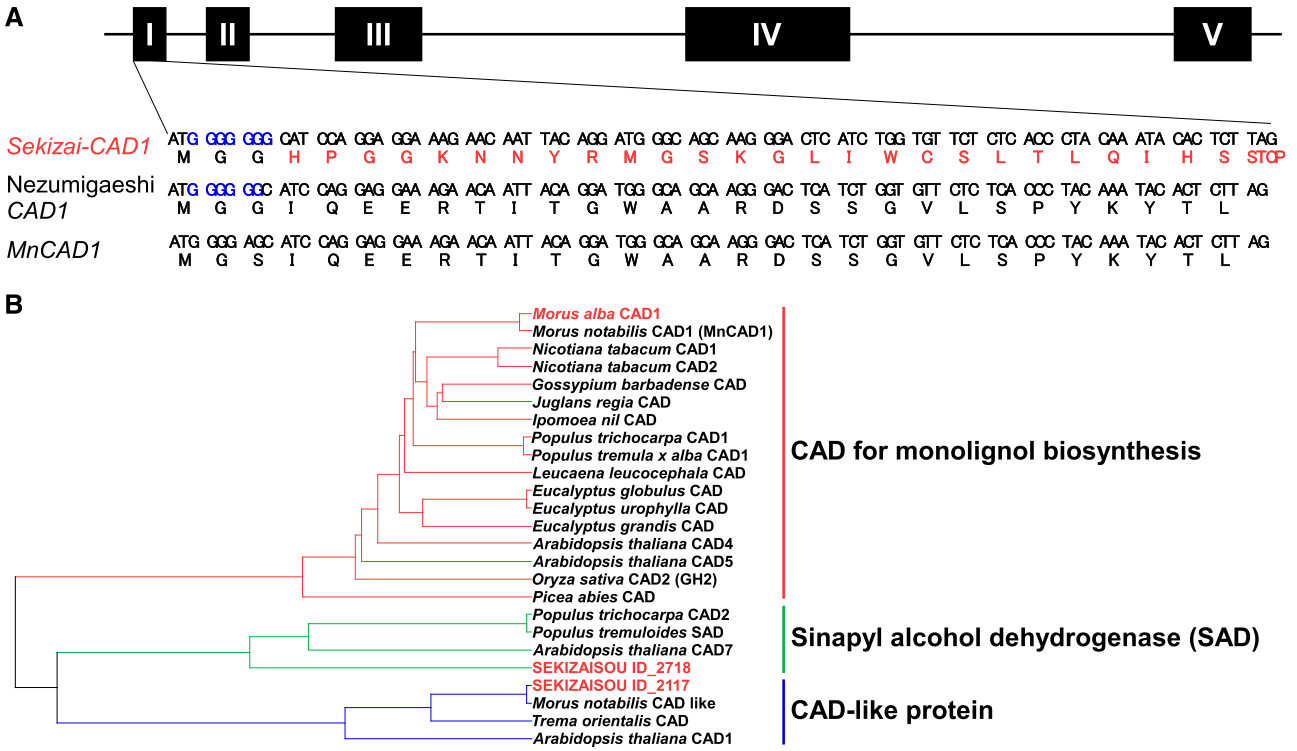

Figure 4. Identification of *CAD1* by draft genome sequencing, de novo assembly, and subsequent BLAST analysis. A, The gene structure of *CAD1* in cv Sekizaisou (*Sekizai-CAD1*), cv Nezumigaeshi, and *M. notabilis* (*MnCAD1*). The genes include five exons (black boxes) and four introns (black lines). Nucleotide sequences of first exons (top line) and the deduced amino acid sequences (bottom line) of the genes are shown. After the first codon (ATG) in the first exon, a guanine hexad (blue) was detected in the sequence of *Sekizai-CAD1*, whereas both *CAD1* alleles in cv Nezumigaeshi contain a guanine pentad. Amino acid sequences deduced from the first 29 codons of *CAD1* in cv Nezumigaeshi and *M. notabilis* were identical, except for the residues encoded by the third codon. The guanine insertion into the first, second, or third codon of the first exon in *Sekizai-CAD1* causes a frameshift mutation and subsequent premature stop codon. B, The phylogenetic tree of CAD sequences in different plant species. The amino acid sequence encoded by *Sekizai-CAD1* was identical to those (319 amino acids) encoded by both entire *CAD1* alleles in cv Nezumigaeshi if the inserted guanine residue was removed from the *Sekizai-CAD1* sequence. The phylogenetic tree was constructed by using the entire CAD1 sequence of cv Nezumigaeshi as the CAD1 sequence of all *M. alba* cultivars.

exon of *Sekizai-CAD1*. A frameshift mutation in *CAD* has been also reported in the pine *cad-null* mutant in which wood color was brown (Ralph et al., 1997; Gill et al., 2003). *Sekizai-CAD1* transcripts of similar size as *CAD1* transcripts in cv Nezumigaeshi were detected by reverse transcription-PCR (Supplemental Fig. S5), indicating that the mutant gene is still actively transcribed. The sequencing results are in line with a homozygous recessive allele in cv Sekizaisou, as predicted from the phenotypes of the F1 progenies derived from crosses between cvs Sekizaisou and Kokusou 21 (Supplemental Fig. S2). To the best of our knowledge, this is the first report on a homozygous mutation in *Morus* with an abnormal phenotype and the first report on an angiosperm lignin mutant, even though the mutation had not been recognized until now.

Accession Numbers

DNA sequences of *CAD1* from cvs Sekizaisou and Nezumigaeshi have been deposited in DDBJ, ENA, and GenBank with the following accession numbers: LC476972 (*Sekizai-CAD1*), LC476973 (*allele 1* in cv Nezumigaeshi), and LC476974 (*allele 2* in cv Nezumigaeshi).

Supplemental Data

The following supplemental materials are available.

Supplemental Materials and Methods. Detailed information on materials and methods.

Supplemental Figure S1. An ancient photograph of cultivated Sekizaisou.

Supplemental Figure S2. cv Sekizaisou and its F1 progenies.

Supplemental Figure S3. Structures of the main thioacidolysis products.

Supplemental Figure S4. Mass spectra of lignin-derived indenes.

Supplemental Figure S5. Exon-intron structure of *CAD1* genes in *Morus* sp.

Supplemental Figure S6. Molecular structures of differentially accumulated metabolites.

Supplemental Table S1. Lignin content and monomeric composition.

Supplemental Table S2. Methanol-soluble phenolics differentially accumulated between cv Sekizaisou and other cultivars.

Supplemental Table S3. List of cultivars used for metabolomic analysis.

Supplemental Table S4. Nucleotide sequences of PCR and sequencing primers.

Supplemental Table S5. Full list of peaks detected in the cv Sekizaisou and other cultivars.

ACKNOWLEDGMENTS

We thank Dr. Gilles Pilate and Dr. Annabelle Déjardin (Institut National de la Recherche Agronomique) for providing the transgenic poplar plants, and National Agriculture and Food Research Organization Genebank for the distribution of mulberry cultivars. We would also like to express our gratitude to Shinta Inagaki, Dr. Hiroaki Yamanouchi, Dr. Akemi Shimizu, and Takeshi Yokoyama (Okushiri Board of Education) for providing the information on cv Sekizaisou. Dr. Yasuhiko Matsushita is acknowledged for technical support in analysis of the genome sequences.

Received November 27, 2019; accepted January 31, 2020; published February 12, 2020.

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