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Note

Laticifers in mulberry exclusively accumulate defense proteins related to biotic stresses

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Abstract A laticifer is an elongated tubular cell and its cytoplasmic content (latex) is thought to be involved in defense against herbivores and microbes. Previous studies investigated laticifer transcriptomes in the unlignified and lignified tissues of mulberry (*Morus alba*) but protein accumulation in laticifers in unlignified and lignified tissues is poorly understood, except the conclusion of a previous study that insecticidal chitinase-like proteins (LA-a and b) were abundant in the laticifers of unlignified tissues while antifungal class I chitinase (LA-c) was abundant in the laticifers of lignified tissues. In order to understand precisely the physiological roles of laticifers in these tissues, this study identified the major proteins in them using mass spectrometry and Edman sequencing after separation by ion-exchange chromatography and SDS-PAGE. In addition to LA-a, b and c, this study has shown that mulberry laticifers accumulate large amounts of biotic-stress-related defense proteins, e.g., pathogenesis-related protein-1, β-1,3-glucanase, class V chitinase, osmotin and lectins. The abundance of some proteins varied among the laticifers of unlignified and lignified tissues, which suggested that the laticifers may have adapted to different threats.

Key words: Latex, laticifer, lectin, proteome, PR protein.

A laticifer is a unique cell that forms an elongated tubular and branched network that runs throughout a plant body and, due to this structure, large amounts of its cytoplasm is exudated when the plant body is cut (Hagel et al. 2008). Laticifer cytoplasm, called latex, contains toxic compounds or proteins, which are thought to be involved in defense against herbivores and microbes (Hagel et al. 2008; Konno 2011). Laticifers are not found in model plants, such as Arabidopsis and rice, but are estimated to exist in 20,000 species in 40 families (Lewinsohn, 1991). Previous studies found that two insecticidal chitinase-like proteins, named LA (Latex-abundant) -a and b, significantly accumulated in laticifers of the green-colored, unlignified tissues of mulberry (*Morus alba*, *Moraceae*), while antifungal class I chitinase (LA-c) was abundant in laticifers located in the brown-colored, lignified tissues (Kitajima et al. 2010, 2012). The mRNA-seq analysis indicated that the mRNAs of various possible biotic-stress-related proteins, as well as the three LA proteins, were highly abundant in mulberry laticifers (Kitajima et al. 2012). However, this study found that mRNA levels were not always consistent with protein levels in laticifers. The mRNA of a hypothetical protein, which has a similar sequence to LA-a and b, was the most abundant mRNA in laticifers found in lignified tissue, but its protein product was not detected in these tissues (Kitajima et al. 2012). Moreover, a BURP domain-containing protein, whose homologs in other plant species were suggested to be stress-related according to the accumulation pattern of their mRNAs (Ding et al. 2009; Kim et al. 2009; Lal et al. 2009; Shin et al. 2009; Xu et al. 2010), was not detectable in mulberry laticifers when analyzed using polyclonal antibodies raised against its recombinant protein (unpublished results), although its transcript was abundant (the 11th and 7th ranks in laticifers of unlignified and lignified tissues, respectively) (Kitajima et al. 2012). Therefore, in order to precisely understand the physiological roles of these laticifers, this study investigated the proteins that were abundant in mulberry laticifers.

Latexes of unlignified and lignified tissues were obtained from the soft green-colored tissues near the stem tip of mulberry (cv. Minamisakari) and from the hardened brown-colored trunk consisting of tissue that was over 2 years old, respectively. Latex exuded from cut sites was rapidly mixed with an equal volume of buffer A

Abbreviations: LA, Latex-abundant; MS, mass spectrometry; PR, Pathogenesis-related.
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(100 mM potassium phosphate and 10 mM EDTA at pH 6.7) supplemented with 0.1% (v/v) β-mercaptoethanol, frozen in liquid nitrogen and stored at −80°C. A total of 9 ml of the latex/buffer A mixture was diluted four times with buffer A and centrifuged at 18,000 *g* for 30 min. The supernatant was filtered successively through 5, 0.80, and 0.45 μm nitrocellulose filters to remove any latex particles and then the proteins were precipitated using 80% saturated ammonium sulfate. The precipitated proteins were dissolved in 5 ml of buffer B (10 mM potassium phosphate and 1 mM EDTA at pH 7.0) and desalted by being passed twice through a PD10 column (GE Healthcare, Piscataway, NJ, USA) equilibrated with buffer B. The eluent was mixed with approximately four times the eluent volume of ultrapure water and loaded onto a Q-sepharose (GE Healthcare) anion-exchange column (26 mm i.d.×100 mm) equilibrated with buffer B. The absorbed proteins were then eluted by 0.05, 0.1, 0.15, 0.2, 0.3 and 1.0 M KCl. The flow-through fraction, containing the unabsorbed proteins, was loaded onto a SPXL (GE Healthcare) cation-exchange column (26 mm i.d.×100 mm) equilibrated with buffer B and then eluted using a step-gradient of increasing KCl. The eluted proteins were separated by SDS-PAGE.

Figure 1A and B show the results for the laticifers of unlignified and lignified tissues, respectively. LA-a, LA-b (Figure 1A) and LA-c (Figure 1B) were the most abundant proteins in the laticifers and had been identified previously (Kitajima et al. 2010, 2012). LA-a is a protein equivalent to MLX56 reported by Wasano et al. (2009). Another ten and eight abundant proteins from unlignified and lignified laticifers, respectively, were selected in order to identify the protein species.

The protein bands were *in-gel* digested by trypsin (Promega, Madison, WI, USA). MS and MS/MS spectra of the digests were obtained using Autoflex speed TOF/TOF (Bruker Daltonics, Bremen, Germany). These were compared to the database using the Mascot program (Matrix Science, Boston, MA, USA). A sequence database of laticifer mRNAs for mulberry (DDBJ/GenBank/EBI accession No.FX177585–FX178648 and FX179391–FX180059) was constructed according to the mRNA-seq analysis (Kitajima et al. 2012). For confirmation, amino acid sequences of the proteins were analyzed by Edman degradation using a protein sequencer, PPSQ-30 (Shimadzu, Kyoto, Japan). For N-terminal amino acid sequencing, each protein band was blotted onto a polyvinylidene fluoride membrane. For internal amino acid sequencing, the protein band was *in-gel* digested by Glu-C (Roche Diagnostics, Mannheim, Germany) and then the peptides were separated by Tricine-SDS-PAGE and blotted onto a polyvinylidene fluoride membrane. The results are shown in Table 1.

In both types of laticifers, the identified proteins were

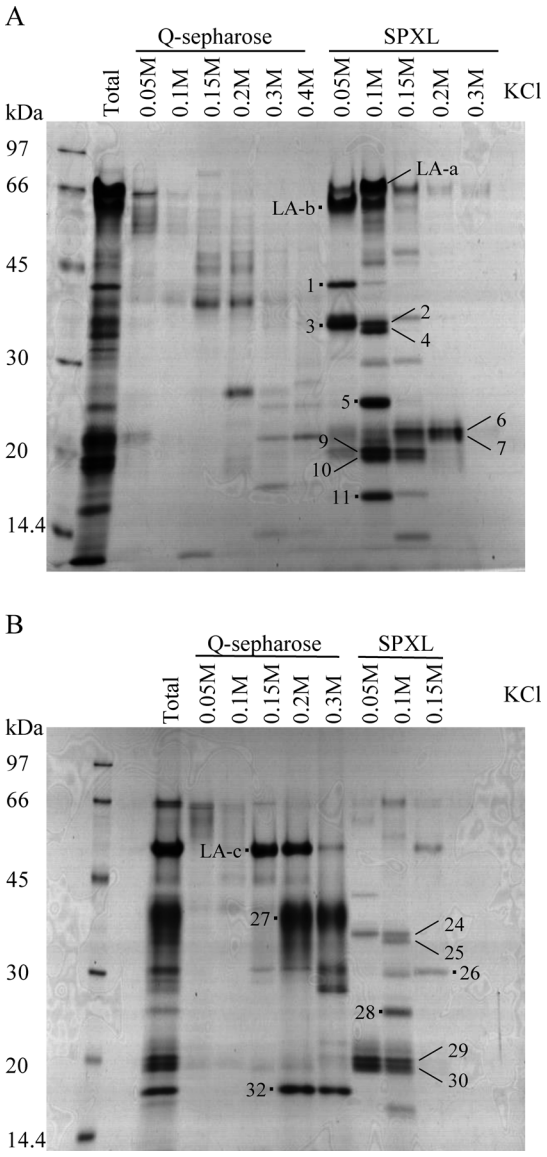

Figure 1. SDS-PAGE of the laticifer proteins found in the soft green-colored tissues near the stem tip (unlignified tissue, A) and in the hardened brown-colored trunk consisting of tissue over 2 years old (lignified tissue, B). Proteins were fractioned by step-gradient Q-sepharose anion-exchange chromatography. The proteins in the flow-through were fractioned by step-gradient SPXL cation-exchange chromatography. The sample total is the total before it was loaded onto the Q-sepharose column. The proteins were stained by Coomassie Brilliant Blue G-250.

products of mRNAs ranked in the top 30 most abundant mRNAs by a previous study (Kitajima et al. 2012), except for one of the chitinases (band 1) and pathogenesis-related (PR) protein-1 (band 11). They were all possible defense proteins against insects or microorganisms (Kitajima and Sato 1999; Ma et al. 2010; Subramanyam et al. 2008; van Loon et al. 2006), which is consistent with the idea that laticifers are specialized for defense against biotic stresses (Kitajima et al. 2010, 2012; Konno 2011).

In the unlignified tissue laticifers, one of the galactose-binding lectins (bands 6 and 7) and PR-1 (band 11) were

Table 1. Abundant laticifer proteins in unlignified and lignified tissues of mulberry.

No	Protein (Accession No.)	MS/MS			Edman degradation
		Peptides matched	Sequence coverage (%)[a]	Mascot score	Sequence
Unlignified tissues					
1	Class V chitinase (AB762077)	6	19	274	Not determined
2	β-1,3-glucanase (AB762078)	4	23	115	Not determined
3	Class I chitinase (AB762079, AB762080)	3	12	101	*N*[c]-EQXGSQAG
4	Class I chitinase (FX178320[b])	3	11	166	*N*-EQXGQQAG
5	Osmotin (AB762081)	5	38	205	*N*-ATFTIRND
6	Lectin, galactose-binding (FX177713[b])	2	9	105	Not determined
7	Lectin, galactose-binding (FX177713[b])	2	9	103	Not determined
9	Lectin, galactose-binding (AB762074)	1	7	120	*I*[d]-XVAFDDG
10	Lectin, galactose-binding (AB762074)	1	7	113	Not determined
11	Pathogenesis-related protein 1 (AB762082)	2	15	109	*I*-VGVGP
Lignified tissues					
24	β-1,3-glucanase (AB762078)	2	8	110	Not determined
25	Class I chitinase (FX178320[b])	4	13	127	Not determined
26	Class I chitinase (FX178320[b])	4	13	168	Not determined
27	Not identified				
28	Osmotin (AB762081)	5	38	188	*N*-ATFTIRND
29	Lectin, galactose-binding (AB762074)	1	7	148	Not determined
30	Lectin, galactose-binding (AB762074)	1	7	141	Not determined
32	Lectin, mannose-binding (AB762075, AB762076)	2	19	233	*I*-XPFEANIP

[a] Percentage of the sequence matched versus the whole of the protein. [b] Sequence obtained by transcriptome shotgun assembly (TSA)(Kitajima et al. 2012) [c] N-terminal amino acid sequence determined by Edman degradation. [d] Internal amino acid sequence determined by Edman degradation.

more abundant (as was LA-a and LA-b) than they were in the lignifed tissues. In contrast, mannose-binding lectin (band 32) was more abundant (as was LA-c) in the lignified tissue laticifers than in the unlignifed tissues (Figure 1A and B).

Unlignified soft organs, such as leaves, where laticifers run through veins, may form part of the diet of *Lepidoptera* caterpillars that cannot eat lignified and hardened branches or trunks, whereas the lignified parts may be the target of pathogenic fungi and bacteria. The different accumulation pattern of lectins and PR-1, in addition to LA-a, b, and c, may be related to different threats faced by these tissues, although there have been no reports on the toxic effects of PR-1 on insects.

Accurate nucleotide sequences for cDNAs encoding the identified proteins, with the exception of one class I chitinase (band 4, 25 and 26) and galactose-binding lectin (bands 6 and 7), were determined by RT-PCR cloning and deposited in the public DNA databases. The accession numbers are shown in Table 1.

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