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Tricin-lignins: occurrence and quantitation of tricin in relation to phylogeny

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SUMMARY

Tricin [5,7-dihydroxy-2-(4-hydroxy-3,5-dimethoxyphenyl)-4H-chromen-4-one], a flavone, was recently established as an authentic monomer in grass lignification that likely functions as a nucleation site. It is linked onto lignin as an aryl alkyl ether by radical coupling with monolignols or their acylated analogs. However, the level of tricin that incorporates into lignin remains unclear. Herein, three lignin characterization methods: acidolysis; thioacidolysis; and derivatization followed by reductive cleavage; were applied to quantitatively assess the amount of lignin-integrated tricin. Their efficiencies at cleaving the tricin-(4′–*O*–β)-ether bonds and the degradation of tricin under the corresponding reaction conditions were evaluated. A hexadeuterated tricin analog was synthesized as an internal standard for accurate quantitation purposes. Thioacidolysis proved to be the most efficient method, liberating more than 91% of the tricin with little degradation. A survey of different seed-plant species for the occurrence and content of tricin showed that it is widely distributed in the lignin from species in the family Poaceae (order Poales). Tricin occurs at low levels in some commelinid monocotyledon families outside the Poaceae, such as the Arecaceae (the palms, order Arecales) and Bromeliaceae (Poales), and the non-commelinid monocotyledon family Orchidaceae (Orchidales). One eudicotyledon was found to have tricin (*Medicago sativa*, Fabaceae). The content of lignin-integrated tricin is much higher than the extractable tricin level in all cases. Lignins, including waste lignin streams from biomass processing, could therefore provide a large and alternative source of this valuable flavone, reducing the costs, and encouraging studies into its application beyond its current roles.

Keywords: thioacidolysis, acidolysis, derivatization followed by reductive cleavage, Poaceae, tricin-d_6, stable isotopically labeled internal standard, liquid chromatography-mass spectrometry, multiple reaction monitoring.

INTRODUCTION

Lignocellulosic biomass is the most abundant sustainable source of feedstocks with the potential to produce fuels, chemicals and composites. It predominately contains three components: the linear polysaccharide cellulose with its highly crystalline structure; branched hemicellulosic polysaccharides with variable and amorphous structures; and the phenylpropanoid polymer lignin. Accounting for 15–30 wt% of biomass material, and an even higher level of the plant's carbon and energy content, lignin is located, at maturity, in the walls of a number of cell types, including sclerenchyma fibers, where it occurs in secondary walls as well as the underlying primary walls and middle

lamella (Harris, 2005; Vanholme *et al.*, 2010). Particularly in grasses (family Poaceae), it has been shown to link to feruloylated arabinoxylans and so strengthen the lignocellulose matrix (Ralph, 2010). Chemically, it is a racemic aromatic polymer composed primarily of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units that are derived from three monolignols: *p*-coumaryl; coniferyl; and sinapyl alcohols, respectively (Freudenberg and Neish, 1968; Ralph *et al.*, 2008). The monolignols are linked together and, more importantly, to the growing polymer, through C–O and C–C bonds by combinatorial radical coupling during lignification to form the complex and heterogeneous lignin polymer (Ralph *et al.*, 2004). Different inter-unit linkages, such as β–*O*–4, 4–*O*–5, β–5, β–1, 5–5 and β–β, are generated during the coupling process. The composition of H, G and S units, and the ratio of different units (characterized by their inter-unit linkages) vary in different plant species, cell wall types, and even the environment in which plants grow (Boerjan *et al.*, 2003).

To characterize the inter-unit structural distribution and quantify the proportion of building units in native lignins, chemical degradation reactions followed by gas chromatographic (GC) or liquid chromatographic (LC) analysis are frequently applied in wood and plant chemistry. They can be classified according to the mechanism underlying the depolymerization of the lignin network, namely oxidative, solvolytic and hydrogenolytic reactions (Lapierre, 2010). Among the various methods, acidolysis (Lundquist and Lundgren, 1972; Lundquist, 1973), thioacidolysis (Lapierre *et al.*, 1985) and derivatization followed by reductive cleavage (DFRC; Lu and Ralph, 1997) are commonly used to depolymerize lignin based on the cleavage of aryl ether bonds that represent some 50% of all inter-subunit bonds in softwood lignins and 65–80% in hardwood lignins (Brunow and Lundquist, 2010).

Tricin [5,7-dihydroxy-2-(4-hydroxy-3,5-dimethoxyphenyl) -4H-chromen-4-one] is a flavone originating from a combination of the shikimate-derived phenylpropanoid and the acetate/malonate-derived polyketide biosynthetic pathways. It has long been studied as an extractable portion of various grasses and cereals (family Poaceae), such as wheat, bamboo, oat, maize and rice (Markley and Bailey, 1935; Vogel *et al.*, 1962; Harborne and Hall, 1964). The extractable tricin can occur in either free form (Nakano *et al.*, 2011) or in various conjugated forms, such as tricin-glycosides (Duarte-Almeida *et al.*, 2007; Van Hoyweghen *et al.*, 2010; Bottcher *et al.*, 2013), tricin-lignans (Bouaziz *et al.*, 2002; Wenzig *et al.*, 2005) and (tricin-glycoside)-lignans (Bottcher *et al.*, 2013; Lee *et al.*, 2015), as shown in Figure 1. As we recently reported, not all of the tricin-lignans may be such (Lan *et al.*, 2016); as the ones we extracted for metabolite profiling displayed no optical activity and were obviously the result of a remarkable variety of combinatorial coupling products from not just the monolignols but the monolignol acetate and *p*-coumarate conjugates, we contended that these are incipient tricin-lignins or tricin-(oligo)lignols or, more generally, flavonolignins or flavonolignols (Lan *et al.*, 2016). These compounds are believed to protect plants from pathogens (Park *et al.*, 2007), even though the physiological function of tricin in plants remains poorly understood. The biological activities of tricin, which have been reported recently, include its antioxidant, anti-aging, anticancer and cardioprotective potential (Oyama *et al.*, 2009; Zhou and Ibrahim, 2010; Chambers *et al.*, 2015). Tricin is therefore considered to be a valuable compound for human health.

Recently, tricin was disclosed to be presented in lignins isolated from wheat straw via its characteristic correlations in short-range 2D ^{1}H–^{13}C coherence (HSQC, heteronuclear single-quantum correlation) NMR spectra (del Río *et al.*, 2012). A follow-up study using biomimetic radical coupling reactions confirmed the existence of this structure in polymeric lignin and implicated tricin as a possible nucleation site for lignification (Lan *et al.*, 2015). Preliminary estimates of its concentration as being as high as 15% of wheat straw lignin, as determined by contour volume-integration in HSQC spectra (Zikeli *et al.*, 2014), were thought to be excessive; it is well known that end-groups are overquantified by such methods that are semi-quantitative at best (Mansfield *et al.*, 2012). The actual amount of tricin on the lignin polymers remains unclear, and a method to accurately quantify tricin levels is required in order to perform a systematic study of tricin functionalization across plant species. Given that tricin only attaches to lignin polymers via tricin-4′–*O*–β-ethers, several β-ether-cleaving lignin characterization methods (acidolysis, thioacidolysis and DFRC) might cleave tricin from the lignin matrix for quantitation. However, the chemical reaction behavior of tricin under the reaction conditions by which it might be released is unknown.

In this study, we used tricin and the tricin-(4′–*O*–β)-coniferyl alcohol coupling product [T-(4′–*O*–β)-G] as model compounds to evaluate the reaction efficiency of acidolysis, thioacidolysis and DFRC. In addition, a hexadeuterated tricin (tricin-d_6) was synthesized and used as a stable isotopically labeled (SIL) internal standard that is ideal for quantitation, and compensating for the variation of tricin in the reaction, workup and even the detection process (Schäfer *et al.*, 2015). We describe the application of an optimized method to screen a range of seed-plant species and determine the content of tricin on a whole-cell-wall basis and on a lignin basis, comparing the levels with those of freely extractable tricin. We also delineate the distribution of tricin in acetylated lignin gel-permeation chromatography (GPC) fractions to provide insight into the timing of tricin supplied to the lignifying zone and whether it affects the polymerization.

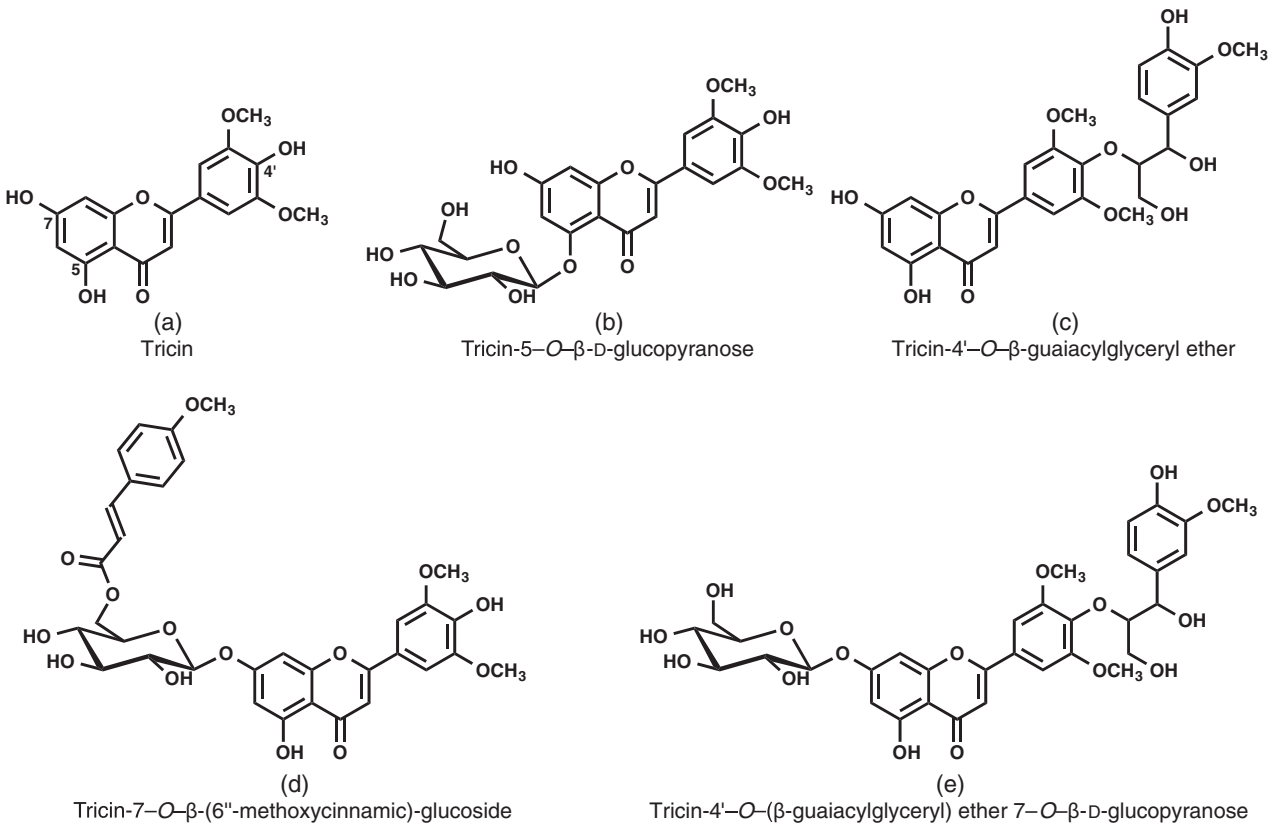

Figure 1. Chemical structures of tricin and example native derivatives.
(a) Tricin.
(b) Tricin-glucoside (Van Hoyweghen *et al.*, 2010).
(c) Tricin-lignan (Bouaziz *et al.*, 2002).
(d) Tricin-(acyl-glucoside) (Duarte-Almeida *et al.*, 2007).
(e) (Tricin-glucoside)-lignan (Lee *et al.*, 2015).

RESULTS AND DISCUSSION

Synthesis of tricin-d_6 as an internal standard

An initial attempt to measure tricin via GC-mass spectrometry (GC-MS) was unsuccessful. Although the volatility of the compound can be improved by trimethylsilylation, the per-trimethylsilyl (TMS) tricin (with TMS on the 5, 7, and 4′ hydroxyl groups) was not stable under the GC conditions and partially degraded into di- and mono-TMS tricin, increasing the complexity for quantitation. Therefore, a LC system equipped with a triple-quadrupole mass spectrometer was employed to quantify tricin using multiple reaction monitoring (MRM). This approach has superior selectivity and sensitivity for quantitation, and does not require any derivatization.

For an accurate quantitative procedure, a suitable internal standard is necessary. The best internal standards usually are SIL analogs because they are nearly identical to the target analyte in chemical and physical properties. As it is processed along with the analyte, the SIL internal standard can not only correct for sample variations during extraction and chemical derivatization, but compensates for the variability in chromatographic separation, ionization and MS detection (Stokvis *et al.*, 2005; Schäfer *et al.*, 2015). We therefore synthesized multiple-deuterated tricin as the internal standard.

Our first attempt was to exchange the five aromatic and alkene protons on tricin via D_2O and Amberlyst® 15 at 110°C (Figure 2). Tricin-d_5 was successfully obtained after a 60 h reaction. However, these deuteriums exchanged with protons under the acidic conditions of acidolysis, thioacidolysis and DFRC. Thus, an acid-tolerant isotope-labeling scheme, deuterating the two aromatic methoxyls, was proposed (Figure 2). The isotopically labeled methoxy-d_3 groups were introduced by nucleophilic substitution of 3,5-di-iodo-4-hydroxybenzaldehyde using $NaOCD_3$ with a catalytic amount of CuBr, thus obtaining the starting material for the synthesis of tricin-d_6 via reaction with 2,4,6-trihydroxyacetophenone as previously reported (Lan *et al.*, 2015).

Evaluation of chemical degradation methods

Acidolysis, thioacidolysis and DFRC are chemical degradation methods commonly used to aid in the determination of lignin structure based on their ability to cleave the aryl ether bond. To quantify the efficiency of these methods at cleaving the tricin-4′–*O*–β bonds and to establish if the tricin remains intact, we ran the three assays on tricin and the T-(4′–*O*–β)-G dimer model compounds, with and without tricin-d_6 as internal standard, producing products as illustrated in Figure 3. Table 1 shows the yields of tricin along with the difference in recovery of tricin-d_6 between adding it before and after the reaction; such a comparison indicates the true yield of tricin in the latter case, and the level accounting for degradation reactions in the former. Tricin was produced from T-(4′–*O*–β)-G dimer in 23% yield with 52% conversion after 0.5 h under acidolysis (entry 3). The conversion was enhanced to 75, 86 and 97%, and the adjusted yield increased to 44, 48 and 52% when the reaction time was prolonged to 1.0, 2.0 and 3.0 h, respectively (entries 4–6). When the internal

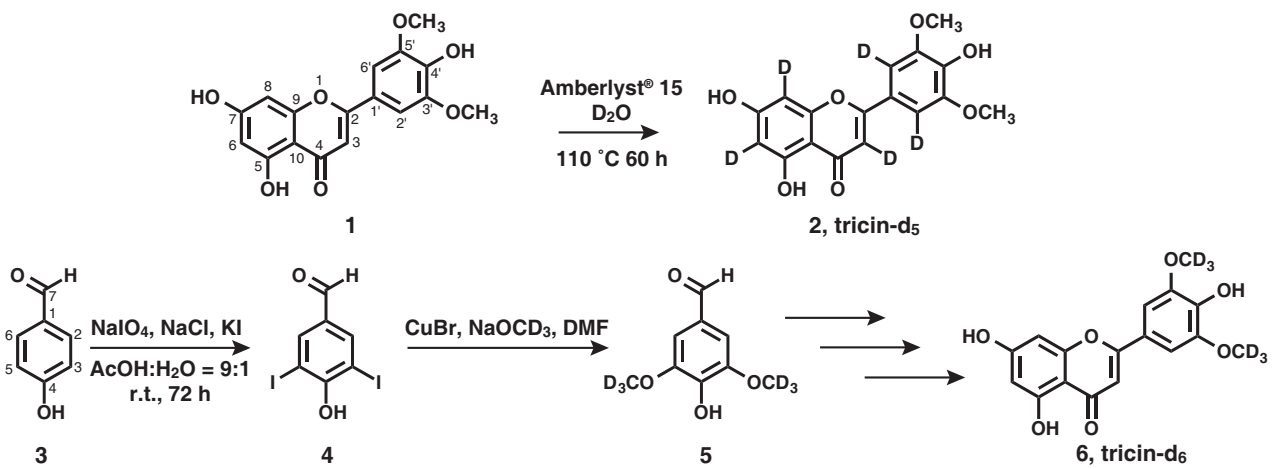

Figure 2. Synthetic scheme for the internal standards (tricin-d_5 and tricin-d_6).

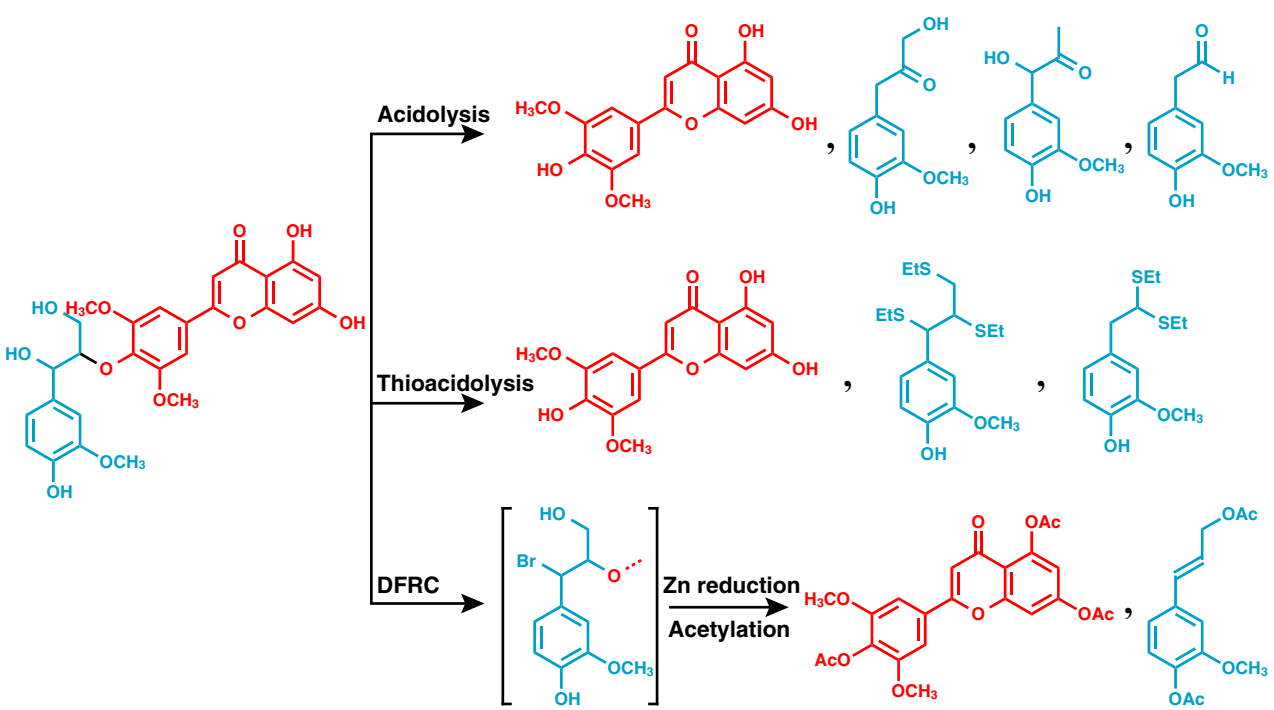

Figure 3. Reaction of tricin-(4′–*O*–β)-coniferyl alcohol [T-(4′–*O*–β)-G] under acidolysis, thioacidolysis and derivatization followed by reductive cleavage (DFRC) conditions for cleaving its 4′–*O*–β-ether bond.

Table 1 Yield of tricin from model compounds via three degradative methods

	Method	Substrate	Conversion[c]	Yield of tricin	Recovery of tricin-d_6	Adjusted yield of tricin[d]
1	Acidolysis, 1.0 h	Tricin[a]	–	81%	88%	93%
2	Acidolysis, 3.0 h	Tricin[a]	–	70%	92%	76%
3	Acidolysis, 0.5 h	T-(4′–*O*–β)-G[a]	52%	23%	88%	26%
4	Acidolysis, 1.0 h	T-(4′–*O*–β)-G[a]	75%	38%	87%	44%
5	Acidolysis, 2.0 h	T-(4′–*O*–β)-G[a]	86%	42%	86%	48%
6	Acidolysis, 3.0 h	T-(4′–*O*–β)-G[a]	97%	44%	85%	52%
7	Acidolysis, 3.0 h	T-(4′–*O*–β)-G[b]	98%	48%	64%	75%
8	Thioacidolysis	Tricin[a]	–	77%	83%	93%
9	Thioacidolysis	T-(4′–*O*–β)-G[a]	100%	84%	87%	96%
10	Thioacidolysis	T-(4′–*O*–β)-G[b]	100%	76%	83%	91%
11	DFRC	Tricin[a]	–	47%	77%	61%
12	DFRC	T-(4′–*O*–β)-G[a]	100%	58%	75%	77%
13	DFRC	T-(4′–*O*–β)-G[b]	100%	55%	45%	120%

[a]Internal standard tricin-d_6 was added after the reaction and before workup.
[b]Internal standard tricin-d_6 was added before the reaction.
[c]Conversion of the substrate = (total substrate − recovered substrate)/(total substrate) × 100%.
[d]Adjusted yield of tricin = yield of tricin/(recovery of tricin-d_6) × 100%.

standard was added before the reaction to track the loss of tricin during the reaction, a recovery-adjusted yield of 75% was obtained after 3 h (entry 7). In the case of using tricin as the substrate, the adjusted yield decreased from 93 to 76% as the reaction time was prolonged from 1.0 to 3.0 h (entries 1 and 2). Moreover, the recovery of internal standard was 85% when it was added after the reaction but before the workup process (entry 6), obviously higher than when it was added at the beginning of the reaction (64%, entry 7), further demonstrating that tricin was degraded under the acidolysis condition. In contrast, tricin exhibited no significant degradation under the thioacidolysis conditions, as shown by the high recovery yield (83%, entry 8) when using tricin as the substrate, and a similar yield for the recovery of the internal standard was found when it was added before (87%, entry 9) and after (83%, entry 10) the reaction. More importantly, the adjusted yield of tricin from the T-(4′–*O*–β)-G flavonolignol was >90% using thioacidolysis (entries 9 and 10). With the DFRC method, tricin suffered more intense degradation than in acidolysis and thioacidolysis, with only a 61% adjusted yield when using tricin as the starting material (entry 11); the recovery of tricin-d_6 was only 45% when added at the beginning of the reaction (entry 13). The adjusted yield of tricin under such conditions was beyond 100%, probably because tricin-d_6 degraded much faster than the covalently bound tricin, resulting in the observed overestimation. Overall, thioacidolysis was found to be the most suitable chemical degradative method to liberate tricin from the lignin polymer due to its greater efficiency at cleaving the tricin-(4′–*O*–β)-ether bond and its ability to keep the liberated tricin intact. This was therefore the method used for our quantitative determinations herein.

Occurrence and content of tricin in the lignins of various seed-plant species

After the first report claiming that tricin was incorporated into the lignin in wheat (*Triticum durum*) straw (del Río *et al.*, 2012), several studies were published showing the occurrence of tricin in lignins from other grass and cereal (family Poaceae) species, such as giant reed (*Arundo donax*; You *et al.*, 2013), rice (*Oryza sativa*; Wu *et al.*, 2013), sugarcane (*Saccharum* sp.; del Río *et al.*, 2015), barley (*Hordeum vulgare*; Rencoret *et al.*, 2015) and maize (*Zea mays*; Lan *et al.*, 2015), as well as in other monocots such as coconut (*Cocos nucifera*; Rencoret *et al.*, 2013). Screening of various seed-plant species and, when present, tricin quantitation, was performed using the above-described thioacidolysis reaction followed by LC-MS characterization. The results, on a cell wall and on a lignin basis (acetyl bromide soluble lignin), are summarized in Figure 4. All samples from species of the family Poaceae (18 samples) contained tricin in their lignins. The amount of tricin was as low as 0.32 mg g^{-1} (cell wall basis) and 1.96 mg g^{-1} (lignin basis) in miscanthus (*Miscanthus* × *giganteus*). The highest level was 7.15 mg g^{-1} (cell wall basis) and 33.11 mg g^{-1} (lignin basis) in oat (*Avena sativa*) straw, with the three highest being detected in oat, wheat and brachypodium (*Brachypodium distachyon*), all of which are in the Poaceae subfamily Pooideae (Kellogg, 2015). Traces of tricin were also found in the lignin of curaua (*Ananas erectifolius*, family Bromeliaceae, order Poales). We extended our survey outside the order Poales and found tricin in the lignin of coconut (*C. nucifera*, family Arecaceae, order Arecales) coir in amounts of 0.26 mg g^{-1} (cell wall basis) and 1.59 mg g^{-1} (lignin basis). Among the eight species in different subfamilies of the family

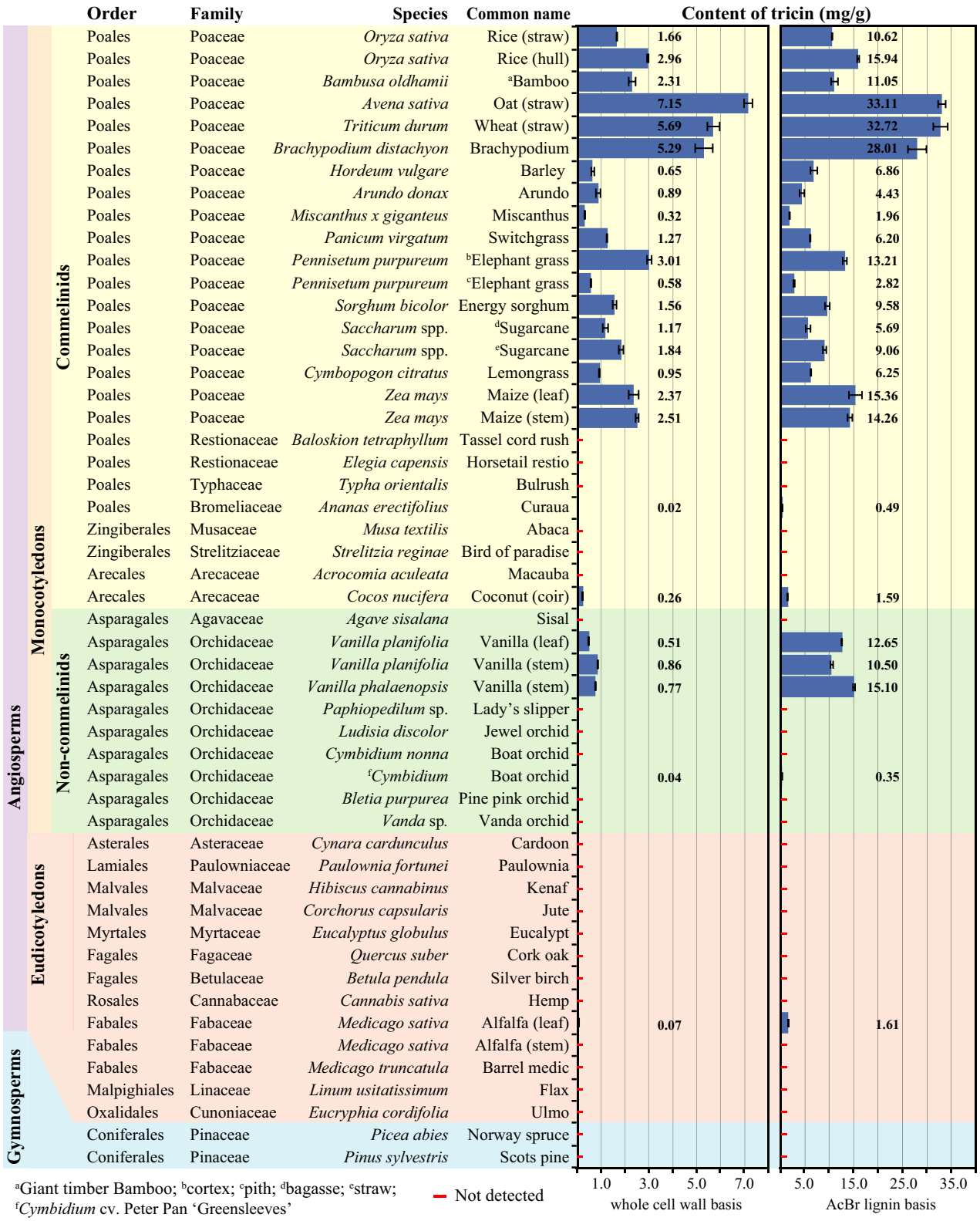

Figure 4. Content of tricin in the lignins from different seed-plant species. The error bars are standard deviations. Extractive-free dried materials were used for measurement by thioacidolysis.

Orchidaceae (non-commelinid, monocotyledon; Chase *et al.*, 2015) that were investigated here, the two species of vanilla investigated, *Vanilla planifolia* and *V. phalaenopsis* (subfamily Vanilloideae), showed moderate amounts of tricin (0.51 and 0.77 mg g^{-1} on leaf cell wall basis, respectively); and the cymbidium Cymbidium cv. Peter Pan 'Greensleeves' (subfamily Epidendroideae) contained traces of it (0.04 mg g^{-1} on cell wall basis). In addition to the monocotyledons, 11 eudicotyledon samples were examined. Only the leaf of alfalfa (*Medicago sativa*) showed the presence of tricin (0.07 mg g^{-1} on cell wall basis and 1.61 mg g^{-1} on lignin basis) in lignin. As this seemed to be an outlier with potentially important implications, we selected another alfalfa sample, carefully hand-selecting the leaves to ensure no contamination, and found the same level upon analysis. No tricin was detected in the two gymnosperm species examined, spruce (*Picea abies*) and scots pine (*Pinus sylvestris*), which is logical given that typical gymnosperms lack syringyl units, due primarily to their lacking a ferulate 5-hydroxylase (Baucher *et al.*, 2003). Overall, the distribution of tricin-lignin in plants is wider than we expected. Although found in all species of grasses examined, it also occurs in other families and orders of commelinid monocotyledons, as well as one species of non-commelinid monocotyledon and one eudicotyledon species. In contrast, *p*-coumarate and ferulate occurs on arabinoxylans (Harris and Hartley, 1980; Ralph *et al.*, 2004; Bunzel, 2010), and *p*-coumarate (and now also ferulate) on/in monocotyledon lignins that are synthesized using monolignol *p*-coumarate/ferulate conjugates in addition to the prototypical monolignols (Ralph *et al.*, 1994; Ralph and Landucci, 2010; Withers *et al.*, 2012; Petrik *et al.*, 2014; Karlen *et al.*, 2016).

Comparison of extractable tricin and lignin-integrated tricin

Tricin has been studied extensively as an extractable compound distributed in various plant species (Li *et al.*, 2016). Its human health benefits have elicited increasing interest in seeking a reliable source of tricin at a lower cost (Oyama *et al.*, 2009; Mohanlal *et al.*, 2011; Moheb *et al.*, 2013). Given its current expense, its interesting potential in agriculture and other fields can scarcely be entertained. In this study, we quantified the contents of free tricin itself, as well as the T-(4′–*O*–β)-G flavonolignol dimer (Lan *et al.*, 2015), and compared the levels with those of integrated tricin (that is incorporated into the lignin polymer) in wheat, maize, oat and rice straw (Table 2). The dried plant samples were ground to fine powders, and extracted several times with 80% methanol by sonicating in an ultrasonic bath. The contents of free tricin in wheat, maize, oat and rice straw were 376, 91, 601 and 64 mg kg^{-1}, respectively (Table 2). The amounts of the T-(4′–*O*–β)-G flavonolignol dimer, another important form of extractable tricin, were 1044, 300, 1270 and 215 mg kg^{-1} in those four species, significantly higher than those of free tricin, and containing 638, 183, 776 and 131 mg, respectively of tricin monomer. Attempting to release tricin from tricin-glycosides via mild acid hydrolysis produced no additional tricin, so we assume that we are measuring most of the extractable

Table 2 Comparison of extractable tricin (MeOH extraction) and lignin-integrated tricin (by analytical thioacidolysis) from non-extracted plant material (mg kg^{-1})

Extractable tricin versus lignin-integrated tricin

Plant sample	Extractable tricin	Extractable T-(4′–*O*–β)-G	Lignin-integrated tricin
Wheat straw	376.1 ± 69.5	1044.3 ± 149.9	4841.3 ± 217.8
Maize straw	90.9 ± 12.4	299.7 ± 39.9	1304.0 ± 39.6
Oat straw	601.4 ± 48.4	1270.0 ± 49.7	5250.3 ± 121.9
Rice stem	64.1 ± 6.9	215.3 ± 28.1	979.7 ± 0.1

Extractable tricin reported in previous studies

Plant species	Part	Extractable tricin	Extractable T-(4′–*O*–β)-G
Njavara rice (*Oryza sativa* cv. Njavara)[a]	Bran	1930.5 ± 0.3	1217.7 ± 1.2
Rice (*Oryza sativa* cv. Sujatha)[a]	Bran	48.6 ± 0.1	45.9 ± 0.9
Rice (*Oryza sativa* cv. Palakkadam Matta)[a]	Bran	119.8 ± 0.1	ND[c]
Wheat (a winter cultivar)[b]	Husk	772 ± 31.8	–[d]
	Leaves	253 ± 18.3	–[d]
	Bran	33 ± 15.9	–[d]

[a]Mohanlal *et al.* (2011).
[b]Moheb *et al.* (2013).
[c]Not detected.
[d]Not reported.

tricin by this method. The highest level of free tricin was reported as 1931 mg kg^{-1}, along with 1218 mg kg^{-1} of T-(4′–*O*–β)-G (containing 744 mg of tricin monomer), which was extracted with methanol from the bran of Njavara rice (*O. sativa* cv. Niavara), a medicinal rice cultivated in India. This content was significantly higher than the non-medicinal rice cultivars Sujatha and Palakkadan Matta with only 49 and 120 mg kg^{-1} of free tricin, respectively (Mohanlal *et al.*, 2011). Wheat husk (winter cultivar), a more affordable source, contained the highest tricin concentration (772 mg kg^{-1}; Table 2) in non-medicinal staple crops and the second highest among plants that have been examined (Moheb *et al.*, 2013). Oyama *et al.* (2009) isolated 8 g of tricin from 40 000 kg of leaves of the bamboo *Sasa albomarginata* by hot water extraction. However, the contents of lignin-integrated tricin were 4841, 1304, 5250 and 980 mg kg^{-1} in wheat, maize, oat and rice stem, respectively, which is higher than for free tricin, even when corrected for the total extractable tricin contents of 1014, 274, 1377 and 195 mg kg^{-1}. More importantly, the differences were much more significant when the tricin level was calculated on a lignin basis (Figure 4). Lignin residues from various biomass pretreatment processes are therefore a potentially attractive alternative economic source of large amounts of tricin if a feasible and industrially relevant method to liberate tricin is available.

Distribution of lignin-integrated tricin in fractions with different molecular weights

To investigate the distribution of tricin in the lignin polymer, acetylated wheat straw lignin was fractionated into 8 molecular weight fractions via preparative GPC. Each fraction was characterized by analytical GPC and short-range ^{1}H–^{13}C NMR correlation (HSQC) spectroscopy. The relative values of *p*-coumarate, tricin, and guaiacyl and syringyl units, as well as β-aryl ether (β–*O*–4), phenylcoumaran (β–5) and resinol (β–β) substructures were calculated based on the corresponding contour volume-integrals in HSQC spectra (Table 3; Ralph and Landucci, 2010; Mansfield *et al.*, 2012). The results of analytical GPC showed that the acetylated wheat straw lignin was successfully divided into eight fractions with different molecular weights, even though the polydispersity became higher in the lower average molecular weight fractions.

The proportion of various units either changed little (such as S/G) or showed no clear trend (such as the *p*CA level), but the two key features showed a trend that was predicted, namely that the higher the tricin level the lower the resinol (β–β) level. Thus, the content of tricin increased with decreasing molecular weight and the resinol percentage showed the opposite trend, decreasing with decreasing molecular weight. As was described in a previous study (Lan *et al.*, 2015), each lignin chain can contain at most one tricin moiety, as tricin can only link with lignin polymers via 4′–*O*-coupling with a monomer. It can therefore be considered to act as a nucleation site in lignification. Likewise, the resinol units can not form within a chain but only from monolignol dimerization, so are also lignin-starting reactions (in the absence of another starting point, such as tricin). They are almost entirely from sinapyl alcohol (or sinapyl *p*-coumarate) dehydrodimerization in monocots and dicots (Lapierre, 1993; Stewart *et al.*, 2009; Ralph and Landucci, 2010). When tricin is present, more of the polymer chains will start by the cross-coupling of tricin with a monolignol than from monomer–monomer coupling. The resinol levels are therefore anticipated to be lower in fractions that have the higher tricin levels. If a polymer chain does start from a β–β-coupled (resinol) unit, that chain can extend from both ends. It is therefore logical that such chains can become longer, on average, than those starting from tricin (although the factors influencing polymer length *in planta* are poorly understood). It is

Table 3 Number-average (Mn) and weight-average (Mw) molecular weights of the wheat straw lignin fractions from GPC and the corresponding relative HSQC volume-integrals from *p*-coumarate (*p*CA), tricin, guaiacyl (G) and syringyl (S) units in the aromatic region, and β-aryl ether (β–*O*–4), phenylcoumaran (β–5) and resinol (β–β) units in the aliphatic region

	1	2	3	4	5	6	7	8
Mn	10 400	8500	7300	5900	4600	4000	2800	1800
Mw	45 200	41 400	38 600	37 400	31 900	29 500	23 600	17 800
Mw/Mn	4.35	4.87	5.28	6.34	6.93	7.38	8.43	9.89
*p*CA	13.7%	13.1%	13.9%	13.7%	13.6%	14.4%	14.8%	15.9%
Tricin	4.5%	4.7%	4.8%	5.0%	5.0%	5.4%	5.6%	6.9%
Guaiacyl	60.2%	60.5%	60.5%	60.7%	61.2%	61.3%	60.6%	61.7%
Syringyl	39.8%	39.5%	39.5%	39.3%	38.8%	38.7%	39.4%	38.3%
S/G	0.66	0.65	0.65	0.65	0.63	0.63	0.65	0.62
β–*O*–4	87.6%	88.3%	87.8%	88.1%	87.5%	88.4%	88.7%	88.9%
β–5	6.0%	5.9%	6.2%	6.3%	6.2%	6.2%	6.5%	6.7%
β–β	6.4%	5.8%	6.0%	5.6%	5.9%	5.4%	4.7%	4.4%

Fractions 1–8 are from preparative GPC. HSQC, heteronuclear single-quantum coherence; GPC, gel-permeation chromatography.

therefore reasonable to expect a relatively higher tricin level in the fractions with lower molecular weight and a relatively higher level of resinols in the fractions with higher molecular weight. The main point, however, is that tricin is found prominently in all molecular weight fractions, suggesting that it does not limit the polymerization and is likely present in the wall throughout lignification.

CONCLUSION

We developed a method that included the use of an SIL internal standard, tricin-d_6, to quantify the amounts of tricin that are covalently linked into the lignin polymer. Thioacidolysis was used to liberate tricin from the lignin matrix for quantitation because of its advantage in not only cleaving the tricin-(4′–*O*–β)-ether bond in high yield but also its lower degradation rate of the released tricin compared with simple hydrolysis or DFRC conditions. The resultant mixture from thioacidolysis was characterized by LC-MS using a triple-quadrupole mass spectrometer and using an MRM scan mode that is superior for low concentration detection and quantification, particularly when used in combination with an isotopically labeled analog as the internal standard. A survey of different seed-plant species for the occurrence and content of tricin showed that tricin is widely distributed in the lignins from monocotyledonous plants, and particularly in the Poaceae family. Among these, oat straw, wheat straw and Brachypodium, all in the subfamily Pooideae, contained the highest levels among the samples we examined, showing 7.15, 5.69 and 5.29 mg g^{-1} on a cell wall basis, and 33.1, 32.7 and 28.0 mg g^{-1} on a lignin basis. Further screening indicated that tricin also occurs in other commelinid monocotyledon families, and in some species of family Orchidaceae (non-commelinid monocotyledons); it was also found in the leaves of at least one eudicot species in the family Fabaceae. Compared with the amount of extractable tricin, the content of tricin that is integrated into lignin was much higher, indicating that lignin, an abundant and affordable material, is a potential source of tricin if a method to economically cleave tricin from the polymer is available. Finally, the study of GPC fractions with different molecular weights showed that the percentage of tricin is slightly higher in lower molecular weight fractions, whereas the content of β–β units was lower, adding evidence to the hypothesis that tricin is acting as a nucleation site for lignification.

EXPERIMENTAL PROCEDURES

General

All chemicals and solvents used in this study were purchased from commercial sources and used without further purification unless otherwise noted. The plant materials were collected from various sources and listed in Table S1. All the plant samples were ground into fine powders (80–100 mesh), and extracted successively three to four times each with water (1:40, g ml^{-1}) and methanol (1:40, g ml^{-1}) to completely remove extractives. After lyophilization, the obtained cell walls were used for quantitation of lignin and tricin. Thin-layer chromatography plates (20 × 20 cm, 1 mm thick, normal phase; Analtech, Newark, DE, USA) were used for raw product fractionation and purification using hexane/ethyl acetate or methanol/dichloromethane as eluent. Flash chromatography was performed using Biotage snap silica cartridges on an Isolera One instrument (Biotage, Charlotte, NC, USA) using a hexane/ethyl acetate (EtOAc) gradient as eluent. Preparative GPC fractionation of an acetylated wheat straw lignin was carried out on a porous styrene-divinylbenzene copolymer column (96 × 5.1 cm) packed with Bio-Beads S-X1 beads (200–400 mesh, 130 g; Bio-Rad, Hercules, CA, USA). The acetylated lignin (600 mg, dissolved in 10 ml of dichloromethane) was introduced onto the top of the column and eluted with dichloromethane under gravity. The high molecular weight exclusion started at approximately 90 min. Eight fractions were collected out to a further 40 min. Each fraction was subjected to analytical GPC and NMR characterization.

Measurements

Nuclear magnetic resonance spectra of synthetic compounds were recorded at 25°C on a Bruker Biospin (Billerica, MA, USA) AVANCE 500 or 700 MHz spectrometer fitted with a cryogenically cooled 5 mm TCI (500 MHz) or QCI (700 MHz) gradient probe with inverse geometry (proton coil closest to the sample). Bruker's Topspin 3.1 (Mac) software was used to process the spectra. The central solvent peak was used as internal reference (δ_C/δ_H: acetone-d_6, 29.84/2.04, dimethyl sulfoxide-d_6, 39.51/2.49, chloroform-d, 77.23/7.24). The relative content of the structural units was via volume-integration of the contours in HSQC spectra; the aromatic area and aliphatic area were calculated separately. In the aromatic region, the relative contents of *p*-coumarate and tricin were calculated based on the sum of syringyl and guaiacyl units. In the aliphatic region, each unit was calculated based on the total of β–*O*–4, β–5 and β–β unit integrals. LC-electrospray ionization (ESI)/atmospheric pressure ionization (API)-MS (Shimadzu LCMS-8040, Columbia, MD, USA) was used to quantify tricin liberated from the whole cell wall materials using the different chemical degradation methods. A Kinetex XB-C18 (250 × 4.6 mm, 5 μm; Phenomenex, Torrance, CA, USA) column was used in the LC system, and eluted with water (solvent A) and acetonitrile (solvent B), each containing 0.1% (v/v) formic acid, using a gradient method (20% B from 0 to 2.5 min, 90% B at 12.5 min, then decrease to 20% B at 12.5 min and 20% B at 20 min). The injection volume was 1 μl; column temperature was 40°C; flow rate was 0.5 ml min^{-1}. Ionization was performed under dual ionization (ESI and API) using a dual ion source, with the nebulizing gas at 2.5 L min^{-1} and drying gas at 15.0 L min^{-1}. Selective detection of tricin was achieved using positive-ion MRM mode. Three transitions of the analyte-associated precursor ion were selected, and the Q1 voltage, collision energy (CE) and Q3 voltage of each transition were optimized. The detailed MRM parameters are listed in Table S2, and the related MS chromatogram and MS spectra are shown in Figure S2. Quantitation of tricin, tricin-d_6 and T-(β–*O*–4)-G was based on the peak area of the precursor ions [tricin m/z 330.70, tricin-d_6 m/z 337.00, T-(β–*O*–4)-G m/z 527.30]; another two transitions were used to further confirm the target analyte. Calibration curves for each compound were developed to calculate the concentrations. Analytical GPC was performed on Shimadzu LC20 with a photo-diode array detector (SPD-M20A). Separation was performed using a series of three columns, first an TSKgel guard column with an Alpha stationary phase (6.0 mm × 4 cm, 13 μm; TOSOH Bioscience, Tokyo, Japan), then a

TSKgel Alpha-2500 column (7.8 mm × 30 cm, 13 μm; TOSOH Bioscience), followed by a TSKgel Alpha-M column (7.8 mm × 30 cm, 13 μm; TOSOH Bioscience). The mobile phase was 0.1 M lithium bromide (LiBr) in *N,N*-dimethylformamide (DMF) at 40°C and a flow rate of 0.3 ml min^{-1}. The molecular weight distribution was calibrated using Polystyrene ReadyCal Standard Set M(p) 250–70 000 (P/N 76552; Fluka, Sigma/Aldrich, St. Louis, MO, USA) at 280 nm. Acetyl bromide lignin absorption spectra were recorded in 1-cm quartz cuvettes using a Shimadzu UV-1800 spectrophotometer.

Preparation and acetylation of wheat straw lignin

Extractive-free wheat straw (700 mg) was milled using a Fritsch pulverizer 7 in 20 ml zirconium oxide jars with 10 × 10 mm zirconium oxide ball-bearings. The milling program was set 600 rpm for 5 min, rest for 5 min and repeat for a total of 15 cycles, reversing direction each cycle, for a total grinding time of 75 min. The resulting powder (2.00 g) was suspended in sodium acetate buffer (45 ml, pH 5.0), inoculated with Cellulysin™ (100 mg; Calbiochem, San Diego, CA, USA) and incubated at 35°C for 72 h. The solids were pelleted by centrifugation (20 min, 11 670 ***g***), the liquids were decanted (and discarded), and the pelleted material was treated with Cellulysin a second time. After the second Cellulysin treatment, the pelleted material was washed three times with RO water (45 ml, ultrasonication 10 min, pelleted by centrifugation). After lyophilization of the pelleted material, the enzyme lignin was extracted twice using 96% dioxane (v/v, 1:50 g ml^{-1}) at ambient temperature for 48 h. The solvent was combined and concentrated under reduced pressure at 45°C, followed by precipitation into acidic water (pH 2.0, 20-fold by volume). The precipitate was collected by centrifugation, washed with distilled water (3 × 50 ml), and lyophilized to produce a cellulolytic enzyme lignin (CEL) at approximately 9 wt% yield (cell wall basis). For acetylation of the CEL, 500 mg of the substrate was dissolved in 10 ml of pyridine/acetate anhydride (10 ml, 2:1, v/v) and stirred at room temperature overnight (approximately 12 h). Acetylated wheat straw lignin was obtained after co-evaporation of the solvent with ethanol under reduced pressure at 45°C until pyridine and acetic anhydride were completely removed.

Acetyl bromide soluble lignin assay

The contents of lignin in the different plant samples were measured by the acetyl bromide lignin method essentially as previously described (Fukushima and Hatfield, 2004). The extract-free biomass material (2–4 mg) and 0.5 ml acetyl bromide solution (25%, in acetic acid, v/v) were added into a 5-ml vial, capped and placed in a heating block at 50°C for 2 h. The mixture was then quantitatively transferred to a 10-ml volumetric flask containing 2 ml NaOH (2 M) and 0.35 ml hydroxylamine (0.5 M). The flask was made up to the volume mark with acetic acid. Absorption at 280 nm of the resulting solution was determined on a UV/Vis spectrophotometer. The lignin content was calculated using the following equation: lignin (wt%) = $(AV)/(\varepsilon\ L\ m)$, in which A is the absorbance at 280 nm; *V* (ml) is the volume of the volumetric flask; *L* (cm) is the cuvette path; *m* (mg) is the mass of sample; ε (L g^{-1} cm^{-1}) is the extinction coefficient, which is 20 in this study (Monties, 1990).

Syntheses of model compounds and internal standard (tricin-d_5 and tricin-d_6)

Synthesis of tricin-(4′–*O*–β)-coniferyl alcohol was reported in a previous study (Anderson *et al.*, 2015). Pentadeuterated tricin, tricin-d_5 (Figure 2), was synthesized by catalyzed deuterium exchange using Amberlyst® 15 in D_2O. Synthesis of hexadeuterated tricin, tricin-d_6 (Figures S1 and 2), began with iodination of 4-hydroxybenzaldehyde **3**, followed by substitution of the iodine with deuterated sodium methoxide ($NaOCD_3$) to produce 3,5-dimethoxy-4-hydroxybenzaldehyde-d_6 **5**, which is the starting material used to synthesize tricin after coupling with 2,4,6-trihydroxyacetohenone, as previously reported (Lan *et al.*, 2015). The [$(M-H)^-$] was calculated as 335.1043 and measured as 335.1045. The detailed procedures for producing compounds **2, 4** and **5** are described below. All ^1H and ^{13}C NMR data of newly synthesized compounds are listed in Data S1.

*Compound **2** (tricin-d_5).* A tube containing tricin (10 mg, 0.03 mmol), Amberlyst® 15 (2 mg) and D_2O (10 ml) was tightly sealed and then placed in a heating block (110°C). The reaction mixture was kept stirring for 48 h. After the reaction, D_2O was evaporated and acetone was added to dissolve the product. The catalyst was filtered off and the filtrate was collected. After evaporation of acetone, tricin-d_5 was obtained quantitatively as light yellow powder.

*Compound **4** (3,5-diiodo-4-hydroxybenzaldehyde).* Iodination of 4-hydroxybenzaldehyde followed the method previously reported (Emmanuvel *et al.*, 2006) with slight modification. To a mixture of 4-hydroxybenzaldehyde (5.00 g, 41 mmol), $NaIO_4$ (8.76 g, 41 mmol) and NaCl (2.39 g, 41 mmol) in 150 ml of acetic acid:H_2O (9:1, v/v) was slowly added KI (6.80 g, 41 mmol). The mixture was stirred at room temperature for 72 h, then it was poured into 500 ml of ice-cold water. Dichloromethane (CH_2Cl_2, 3 × 200 ml) was used to extract the iodized product. The combined organic phase was dried over anhydrous magnesium sulfate ($MgSO_4$), concentrated under reduced pressure, and precipitated in distilled water (500 ml). The 3,5-diiodo-4-hydroxybenzaldehyde was obtained after filtration and lyophilization as white powder with 91% yield.

*Compound **5** (3,5-dimethoxy-4-hydroxybenzaldehyde-d_6)* To substitute the iodines on the aromatic ring with deuterated methoxyls, a saturated sodium methoxide-d_6 ($NaOCD_3$) solution was prepared by dissolving sodium (3.3 g) in methanol-d_4 (6 ml). To a mixture of compound **2** (5.00 g, 13 mmol) and CuBr (0.29 g, 2 mmol) in 50 ml of DMF, 3.90 g of the freshly prepared $NaOCD_3$ solution was added. The mixture was heated to 110°C and stirred for 8 h. After cooling and evaporation of DMF under reduced pressure, 100 ml of acidic water (pH 2.0) was added. The crude product was extracted with ethyl acetate (EtOAc, 3 × 50 ml). The combined EtOAc phase was dried over anhydrous $MgSO_4$ and concentrated, then subjected to flash chromatography for purification. Compound **5** was obtained in 76% yield.

Chemical degradative methods for quantitation of tricin

Acidolysis, thioacidolysis and DFRC were used to degrade the lignin polymer to release tricin for quantitation. A weighed quantity of tricin-d_6 was added as internal standard, at the very beginning of the reaction or right before the workup process, to track the loss and adjust the yield of tricin. Each sample was run in duplicate or triplicate. The detailed procedure was performed as follows.

Acidolysis. T-(4′–*O*–β)-G (1 mg) and 1 ml dioxane containing 0.2 M HCl was added to a 10-ml vial in 100°C sand bath for a

specific period. After cooling down to room temperature, the reaction mixture was extracted three times with 2 ml EtOAc/H_2O (1:1, v/v). The organic phases were combined, dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The products were dissolved in MeOH and transferred to a 10-ml volumetric flask. To quantify the content of tricin, the resulting solution was subjected to LC-MS after filtration through a Teflon filter (0.22 μm).

Thioacidolysis. Analytical thioacidolysis was performed essentially as published (Lapierre *et al.*, 1985). The thioacidolysis reagent was prepared by adding ethanethiol (EtSH, 2.5 ml) and boron trifluoride diethyl etherate [BF_3-O(Et)$_2$, 0.625 ml] in a 25-ml volumetric flask. Freshly distilled dioxane was added to the mark. Freshly made thioacidolysis reagent (0.5 ml) and approximately 1 mg (accurately weighed) T-(4′–*O*–β)-G (or 4 ml reagent with 20 mg biomass material) were added to a 10-ml reaction vial. The vial was capped tightly and kept in a heating block for 4 h at 100°C. After cooling in an ice-water bath, the reaction mixture was transferred to a 60-ml separatory funnel, to which 0.25 ml 0.4 M sodium bicarbonate ($NaHCO_3$) solution (2 ml in the case of biomass) was added to adjust the pH value to 7.0, followed by 0.25 ml 1 M HCl (1 ml in the case of biomass) to acidify the mixture to pH 3.0. EtOAc (5 × 10 ml) was used to extract the degraded products. The combined organic solutions were dried over anhydrous $MgSO_4$, and evaporated under reduced pressure at 45°C. The residue was dissolved in MeOH (10 ml) and injected to LC-MS for quantitation of tricin.

Derivatization followed by reductive cleavage. The DFRC method followed basically that previously described (Lu and Ralph, 1997; Karlen *et al.*, 2016). T-(4′–*O*–β)-G (approximately 1 mg) and 1 ml 20% acetyl bromide solution (v/v, in acetic acid) were added in a 10-ml reaction vial and placed in a 50°C heating block for 2.5 h. The solution was then evaporated under reduced pressure at 50°C. The residue was dissolved in 1 ml of dioxane/acetic acid/water (5:4:1, v/v/v) and 200 mg nano-powdered Zn was added. The mixture was vigorously stirred for 40 min and then filtered through a Teflon filter (0.22 μm). The filtrate was extracted with CH_2Cl_2 (5 × 10 ml). The combined organic phases were dried over sodium sulfate (Na_2SO_4), and the solvent evaporated under reduced pressure. Pyridine/acetic anhydride (2:1 v/v, 1.5 ml) was added to acetylate the crude products. After acetylation, the solvents were co-evaporated with EtOH to completely remove the pyridine and acetic anhydride, and the resulting products were dissolved in acetonitrile (25 ml) for LC-MS analysis.

Quantitation of extractable tricin

The whole cell wall sample (non-extracted material, approximately 500 mg) was added into a centrifuge tube with 40 ml methanol/water (4:1. v/v). The mixture was ultrasonicated for 40 min and then centrifuged. The supernatant was collected and the residue was extracted with 80% methanol again. The sonication treatment was repeated four to five times until the supernatant was colorless. The combined supernatants were evaporated under reduced pressure, and the products were dissolved in 25 ml methanol in a volumetric flask for LC-MS analysis.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Synthetic scheme for the internal standard tricin-d_6.

Figure S2. HPLC-MS data. (a) Multiple reaction monitoring (MRM) chromatogram. (b) The corresponding MS spectra. (c) MS spectra of the analyte-associated precursors under optimized collision energies (CE); the m/z values in red are the selected transitions for MRM under a specific CE.

Table S1. Plant materials used for the quantitation of tricin.

Table S2. Parameters for MRM mode.

Data S1. ^{1}H NMR and ^{13}C NMR data of synthetic compounds.

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