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Phenylalanine derived cyanogenic diglucosides from *Eucalyptus camphora* and their abundances in relation to ontogeny and tissue type

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ABSTRACT

The cyanogenic glucoside profile of *Eucalyptus camphora* was investigated in the course of plant ontogeny. In addition to amygdalin, three phenylalanine-derived cyanogenic diglucosides characterized by unique linkage positions between the two glucose moieties were identified in *E. camphora* tissues. This is the first time that multiple cyanogenic diglucosides have been shown to co-occur in any plant species. Two of these cyanogenic glucosides have not previously been reported and are named eucalyptosin B and eucalyptosin C. Quantitative and qualitative differences in total cyanogenic glucoside content were observed across different stages of whole plant and tissue ontogeny, as well as within different tissue types. Seedlings of *E. camphora* produce only the cyanogenic monoglucoside prunasin, and genetically based variation was observed in the age at which seedlings initiate prunasin biosynthesis. Once initiated, total cyanogenic glucoside concentration increased throughout plant ontogeny with cyanogenic diglucoside production initiated in saplings and reaching a maximum in flower buds of adult trees. The role of multiple cyanogenic glucosides in *E. camphora* is unknown, but may include enhanced plant defense and/or a primary role in nitrogen storage and transport.

1. Introduction

Plants produce a vast array of bioactive natural products. These chemicals help defend plant tissues against herbivory and pathogens. Cyanogenic glucosides (α-hydroxynitrile glucosides) are an important and widespread class of such defense chemicals, which have been recorded in over 2500 plant species from diverse taxa. When tissue of these plants is disrupted, HCN gas is enzymatically released from cyanogenic glucosides, which thus act as phytoanticipins affording an immediate chemical defense response to herbivores and pathogens (Møller, 2010). Despite the importance of cyanogenic glucosides to a wide array of plants, they constitute a very small class of bioactive natural products with only 60 or so different structures identified (Bjarnholt and Møller, 2008). This contrasts with terpenoids, for example, where in excess of 40,000 different structures have been elucidated (Bohlmann and Keeling, 2008).

Cyanogenic glucosides are biosynthesized from six different building blocks: the five protein amino acids L-valine, L-isoleucine, L-leucine, L-phenylalanine, L-tyrosine and the non-protein amino acid L-2-(2′-cyclopentenyl) glycine (Zagrobelny et al., 2008). Structural diversity may be limited in part by the channeled nature of the biosynthetic pathway (Møller and Conn, 1980). In *Sorghum bicolor*, for example, conversion of L-tyrosine to the cyanogenic glucoside dhurrin is catalyzed by two cytochrome P450s and a UDPG-glycosyltransferase, which are envisioned to form a multi-enzyme complex (a metabolon) that prevents free diffusion of auto-toxic intermediates (Jørgensen et al., 2005b; Møller and Conn, 1980; Nielsen et al., 2008; Winkel, 2004). As a consequence, much of the structural diversity of cyanogenic glucosides is achieved simply by modification of the sugar moiety by additional glycosylation(s) or galloylation (Fleming, 1999; Ling et al., 2002). Another factor that is relevant to understanding the diversification in structure of cyanogenic glycosides is their possible additional roles in primary metabolism (Møller, 2010), particularly in nitrogen storage and transport (Bjarnholt and Møller, 2008; Jenrich et al., 2007; Jones et al., 2000). For example, when *Prunus serotina* seedlings germinate, the cyanogenic diglucoside amygdalin is transported from the seeds and metabolized to supply nitrogen to the developing seedling without release of HCN to the surroundings (Swain and Poulton, 1994). These and other data have prompted the suggestion that cyanogenic diglucosides serve primarily as transport forms (Sánchez-Pérez et al., 2008; Selmar et al., 1988; Swain and

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Poulton, 1994). In addition, there is evidence that when available nitrogen is in excess of that required for growth it can be stored for future use as cyanogenic glucosides. For example, following a rise in nitrate supply, the concentration of the cyanogenic glucosides linamarin and lotaustralin was shown to increase in the shoot apex of cuttings from six-week-old *Manihot esculenta* plants (Jørgensen et al., 2005a). Similarly, experiments with five-week-old *S. bicolor* plants showed that the biosynthetic machinery for synthesis of the cyanogenic glucoside dhurrin was transcriptionally induced by application of nitrate fertilizer (Busk and Møller, 2002).

Our understanding of the diverse roles of cyanogenic glucosides may be advanced through studies of plants that contain multiple cyanogenic glycosides, including mono- and diglucosides, especially where the relative abundance of these change through development and in response to different environmental conditions. *Eucalyptus camphora* is a plant species that fulfills these two criteria. Firstly, there is evidence that its foliage contains at least five different cyanogenic glucosides, including the monoglucoside prunasin, its epimer sambunigrin, and the diglucoside of prunasin, amygdalin (Neilson et al., 2006). Secondly, it displays strong ontogenetic influence over cyanogenic glucoside biosynthesis with seedlings significantly lower in total cyanogenic glucoside concentration than their adult counterparts, with some seedlings incapable of releasing HCN after six months of growth (Neilson et al., 2006). In this paper, we report a detailed characterization of the cyanogenic glucosides present in the foliar and reproductive tissues of *E. camphora*, revealing three additional cyanogenic diglucosides designated eucalyptosin A, eucalyptosin B and eucalyptosin C and their preferential occurrence in specific tissues. Whole-plant and leaf ontogeny had unique influence on the overall content and relative abundances of the different cyanogenic glucosides.

2. Results

2.1. Identification of cyanogenic diglucosides in E. camphora

Cyanogenic glucosides from *E. camphora* seedling and sapling foliage and adult foliage, flower buds and fruits were extracted and separated using reverse-phase HPLC. Fractionation and elucidation of prunasin, sambunigrin and amygdalin from adult *E. camphora* foliage has been described previously (Neilson et al., 2006). In all extracts except those from seedling foliage, the HPLC separation afforded additional fractions capable of releasing HCN upon the addition of β-glucosidase.

Targeted LC-MS analysis of this fraction in negative mode for the amygdalin pseudomolecular ion $[M+C_1H_1O_2]^-$ at *m/z* 502.1585 (calcd. 502.1555 for $C_{20}H_{27}N_1O_{11}$ $C_1H_1O_2$; $\lambda = -3.0$ millimass units) resulted in three additional, analogous pseudomolecular ion peaks (Fig. 1a). MS^2 fragmentation of the formate adduct of each of these parent ions yielded both glycosidic bond and cross ring bond cleavage. Major observed fragment ions in negative mode for amygdalin and the three unknown diglucosides were *m/z* 323, 221, 179, 161, 131, 119, 113, 101, 89, 71 and 59 (Fig. 1c), corresponding to A_1, A_2, B_1, B_2, and C_1 fragment ions using the nomenclature developed for fragmentation of oligosaccharides (Domon and Costello, 1988; Fig. 1b). Spectra recorded in positive mode were also recorded but not as informative due to lack of diagnostic fragments derived from cross ring bond cleavage.

Amygdalin has been reported to easily isomerise into neoamygdalin (**4**) especially when subjected to alkaline pH (Fig. 2; Nahrstedt, 1975). To investigate whether one of the three unknown cyanogenic glycosides could be neoamygdalin produced as an artifact of the extraction procedure, a standard of neoamygdalin was synthesized by treatment of amygdalin with 5 mM NH_3 (Fischer, 1885; Koo et al., 2005) and subjected to the same LC-MS analysis as the plant extracts. Neoamygdalin did not co-elute with any of the *E. camphora* constituents able to release HCN. This would suggest that the unknown compounds are amygdalin isomers in which the second glucoside moiety is linked differently compared to the β(1 → 6) linkage found in amygdalin and neoamygdalin. Two different approaches were undertaken to determine the nature of the glucosidic linkages present in the three unknown structures.

The first approach involved spectroscopic comparison with authentic standards of likely amygdalin isomers. An amygdalin isomer (R)-mandelonitrile β-sophoroside in which the two glucose residues are coupled by a β(1 → 2) linkage has previously been isolated from *Eremophila maculata* (Syah and Ghisalberti, 1996). Therefore we isolated this compound from *E. maculata* and confirmed its structure by NMR. Comparison by GC–MS and LC-MS analysis showed that (*R*)-mandelonitrile β-sophoroside co-eluted with one of the unknown constituents (peak **II**, Fig. 1a) and gave an identical fragmentation pattern. This confirmed the structural assignment of the unknown as **5**, which we have named eucalyptosin A (Fig. 2). Given there is no known plant source of the amygdalin isomer (*R*)-mandelonitrile β-cellobioside in which the glucose moieties are β(1 → 4) linked, we chemically synthesized this compound and confirmed its structure by NMR. This synthetic standard was analyzed using LC-MS and GC–MS and found to co-elute and possess an identical fragmentation pattern with one of the other unknown cyanogenic constituents in the *E. camphora* extracts (peak **III**, Fig. 1a). We have assigned structure **6** to the second unknown and named it eucalyptosin B (Fig. 2).

An alternative approach was required to determine the nature of the glycosidic linkage in the third unknown amygdalin isomer (peak **IV**, Fig. 1a). This was because its low abundance precluded NMR analyses and a natural or chemical route to a synthetic standard was not known. The approach was based on theoretical predictions and empirical characterization of MS fragmentation of oligosaccharides (see Domon and Costello, 1988; Mulroney et al., 1995). Specifically, MS^2 of the third unknown yielded a proportionally high intensity of fragment ions at *m/z* 113 and 161, which is diagnostic for the presence of a β(1 → 3) linked laminaribiose disaccharide (Mulroney et al., 1995). Thus structure **7** was assigned to the third unknown, named here eucalyptosin C (Fig. 2). Indeed using this approach alone, all four cyanogenic diglucosides present in *E. camphora* could be successfully characterized as harboring β(1 → 2), β(1 → 3), β(1 → 4) and β(1 → 6) linkages, respectively, highlighting the utility of this technique. In a similar manner, a number of MS studies of oligosaccharides have used fragments representing opening and cleavage across the ring of sugar moieties to provide important diagnostic information on linkage positions (Cmelík and Chmelík, 2010; Domon and Costello, 1988; Li and Her, 1998; Mulroney et al., 1995; Wong et al., 1999).

2.2. Leaf- and whole-plant ontogeny and tissue specificity

Prunasin (**1**), amygdalin (**3**), eucalyptosin A (**5**), and the novel compounds eucalyptosin B (**6**) and eucalyptosin C (**7**) were present in all examined tissue types except for seedling leaves at 141 DAS, which possessed only prunasin (Table 1, Fig. 3). Sambunigrin (**2**), the epimer of prunasin, was not quantified in the different tissues due to the inability to discriminate between epimers using LC-MS. It is noteworthy that previous experimentation using GC–MS has shown that 5% of the total cyanogenic glucosides in fully expanded adult leaves of *E. camphora* can be present as sambunigrin (Neilson et al., 2006). The relative abundances of the cyanogenic glucosides varied across the different ontogenetic stages and in the different tissue types. In adult expanding leaves (<50% of maximum size) cyanogenic diglucosides constituted 6% of the total cyanogenic

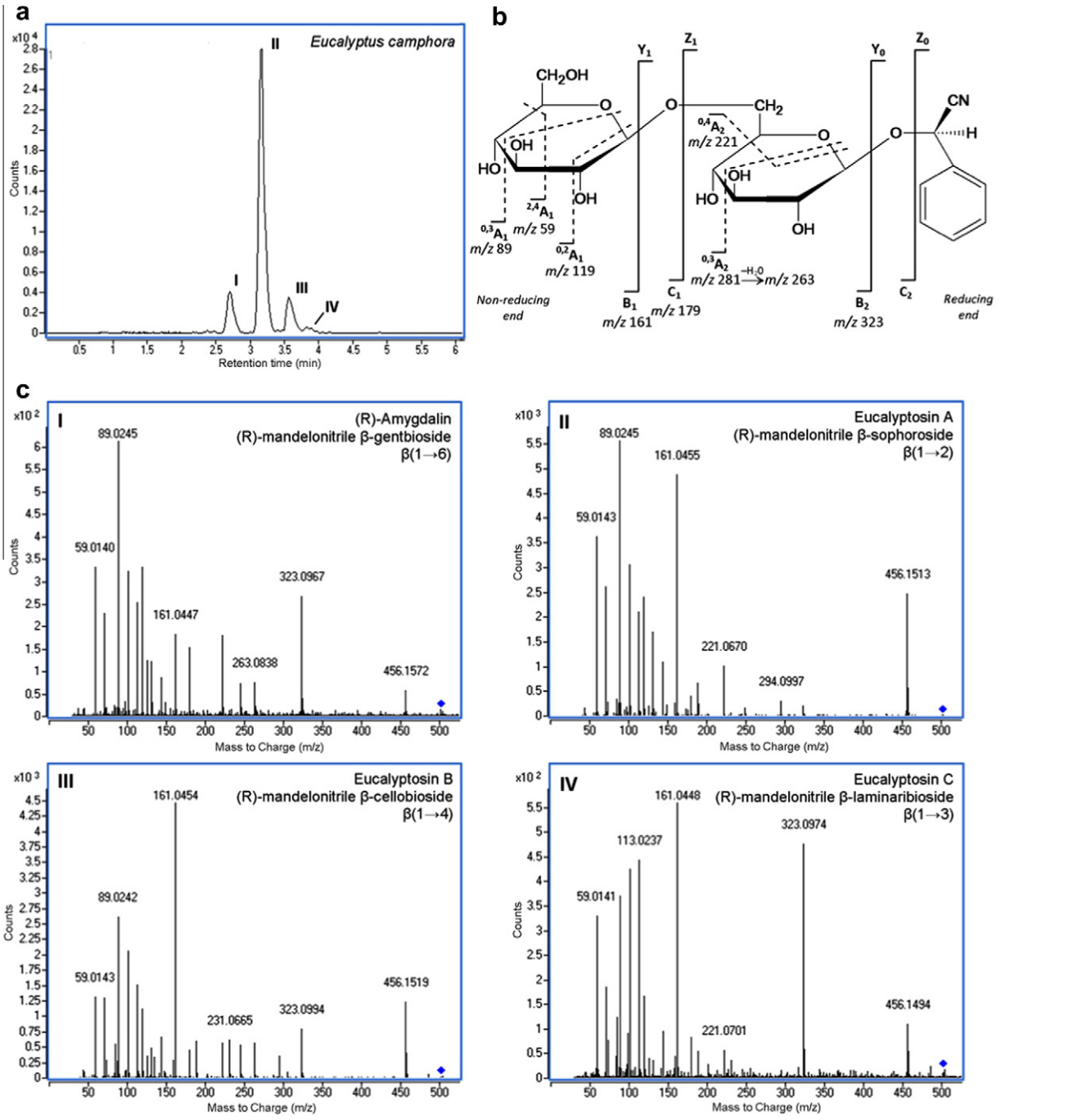

Fig. 1. Elucidation of cyanogenic diglucosides present in *Eucalyptus camphora* extracts using MS. (a) Extracted negative ion chromatograph of *m*/*z* 502.1585 $[M+C_1H_1O_2]^-$ corresponding to the formate adduct of amygdalin reveals four separate peaks. (b) Nomenclature and fragmentation position of glycoconjugate product ions formed by amygdalin (modified from Domon and Costello, 1988). (c) MS^2 fragmentation of the parent ion of each peak (I–IV) yielded ions at *m*/*z* 323, 221, 179, 161, 131, 119, 113, 101, 89 and 59, corresponding to both glycosidic bond and cross ring cleavage, but with distinctly different abundances.

glucoside content. This increased to 17% in adult expanded leaves with the concentration of eucalyptosin A, for example, increasing from 3% to 7% as leaves developed. In flower buds and immature fruits, cyanogenic diglucosides accounted for 27% and 31% of the total cyanogenic glucoside concentration, respectively. In particular, high levels of eucalyptosin A were observed, accounting for over 17% of total cyanogenic glucoside concentration in reproductive tissues. Adult fully expanded leaves and flower buds had similarly high levels of total cyanogenic glucosides, but the total concentration in fruits post-flowering was markedly lower with levels intermediate between moderately cyanogenic saplings and lowly cyanogenic seedlings (Table 1, Fig. 3).

Seedlings of *E. camphora* were repeatedly monitored for the concentration of prunasin in foliage every few weeks until 339 days after sowing (DAS). The onset of prunasin biosynthesis proceeded slowly and was first detected in three of the 57 seedlings at 115 DAS (Fig. 4). As the cohort of seedlings developed, they progressively "switched on" prunasin production, until by 340 DAS all individuals were cyanogenic (Fig. 4a). No relationship between prunasin onset and plant height was observed (data not shown).

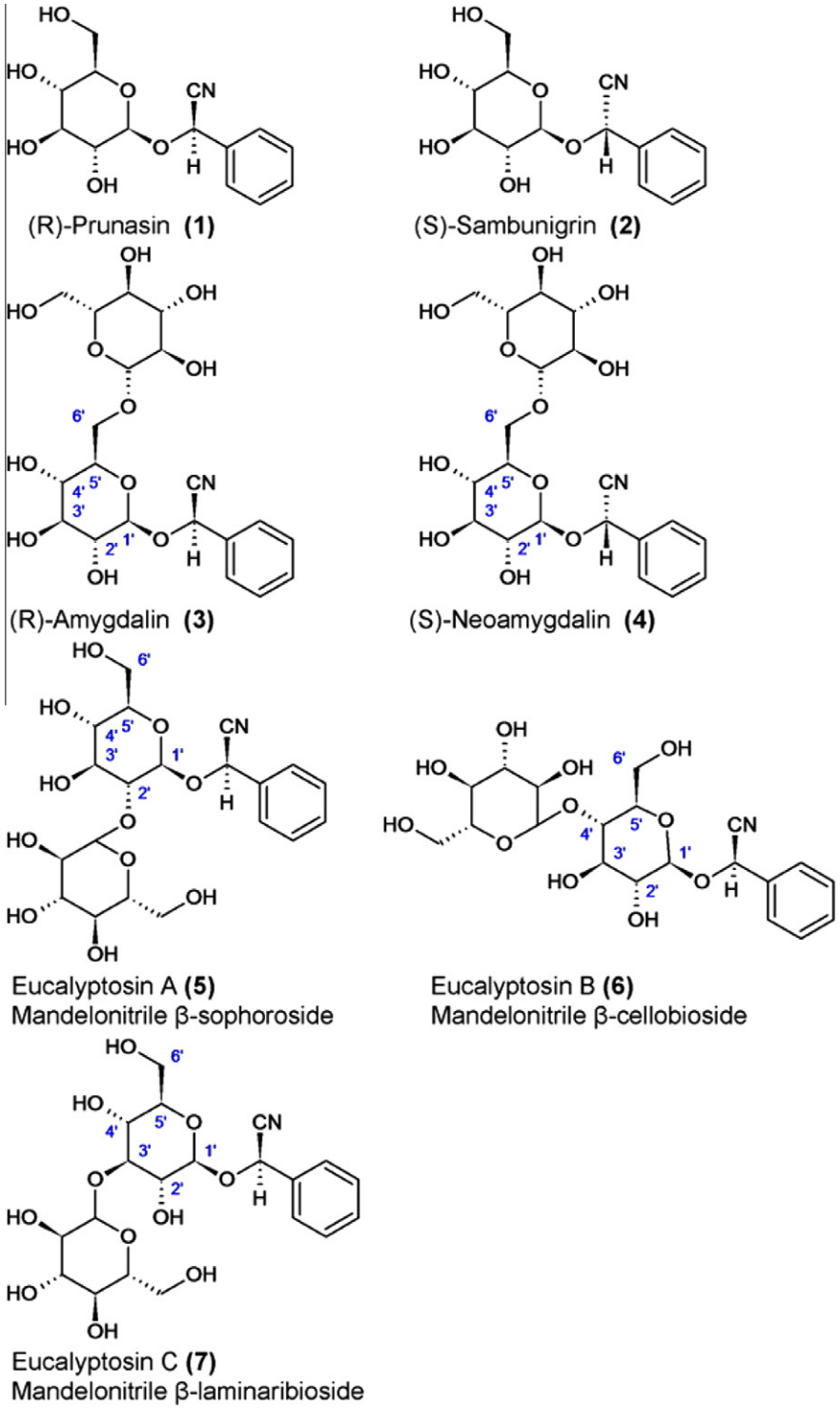

Fig. 2. Structures of phenylalanine derived cyanogenic mono- and diglucosides present in *Eucalyptus camphora*. Prunasin is the most abundant cyanogenic glucoside in foliage.

Once prunasin production was initiated, its foliar concentration in each individual plant increased with time (Fig. 4b). Indeed, examination of the prunasin concentration in sapling leaves (1553 DAS) and adult foliage (Table 1, Fig. 3) suggests prunasin concentration continually increases as plants develop from seedlings, to saplings and then to reproductively mature adult trees.

3. Discussion

3.1. Identification of multiple cyanogenic diglucosides

Three phenylalanine-derived cyanogenic diglucosides were identified in *E. camphora* tissue: eucalyptosin A (**5**), eucalyptosin

Table 1
Total concentration and percentage abundance of the multiple cyanogenic glucosides present in different *Eucalyptus camphora* tissues.

	Prunasin		Amygdalin		Eucalyptosin A		Eucalyptosin B		Eucalyptosin C		Total
	μg g^{-1} fw	%	μg g^{-1} fw	%	μg g^{-1} fw	%	μg g^{-1} fw	%	μg g^{-1} fw	%	μg g^{-1} fw
Seedling expanded leaves (141 DAS)	7	100	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7
Sapling expanded leaves (1553 DAS)	778	85	36	4	41	4	58	6	<1		913
Adult expanding leaves	1806	94	19	1	57	3	29	2	<1		1912
Adult expanded leaves	1619	83	97	5	143	7	93	5	<1		1952
Flower buds	1548	73	45	2	367	17	147	7	<1		2107
Immature fruits	312	69	20	4	87	19	29	6	3	1	451

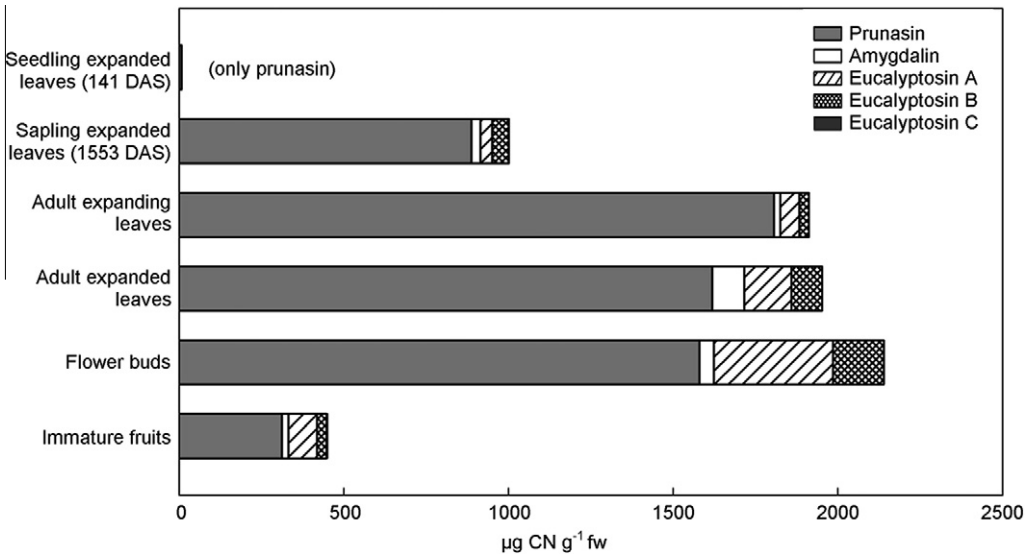

Fig. 3. Cyanogenic glucoside composition at various developmental stages of *Eucalyptus camphora* analyzed in different tissues. Total cyanogenic glucoside concentration and the relative abundances of the mono- and diglucosides differed in the various tissue types. Eucalyptosin C was detected in all sapling and adult tissues examined, but only at very low levels (<1 μg g^{-1} fw).

B (**6**) and eucalyptosin C (**7**), in which the glucose moieties are combined by β(1 → 2), β(1 → 4) and β(1 → 3) linkages, respectively. This is in addition to the previous identification of the phenylalanine-derived monoglucosides prunasin (**1**), and sambunigrin (**2**), and the β(1 → 6) linked diglucoside amygdalin (**3**) in adult leaves of this species (Neilson et al., 2006). Diglucoside **5** has previously been reported from the *E. maculata* (Myoporaceae; Syah and Ghisalberti, 1996) and *Perilla frutenscens* var. *acuta* (Lamiaceae) plants (Aritomi et al., 1985), whereas **6** and **7** are novel compounds. Notably, neoamygdalin (**4**), the epimer of **3**, was not identified in any *E. camphora* tissue.

Structural elucidation of **5** and **6** was based on spectroscopic comparison with authentic standards that were either isolated from other plant species or chemically synthesized. The structural assignment of the lowly abundant **7** was based on the consistency of its MS^2 fragmentation pattern with the empirical criteria for the presence of a β(1 → 3) linkage (Domon and Costello, 1988; Mulroney et al., 1995). In retrospect, it would have been possible to identify the linkage types present within all four *E. camphora* cyanogenic diglucosides using mass spectrometry alone, without the need for NMR analyses. This knowledge should facilitate analysis and structural elucidation of cyanogenic diglucosides from other plant species, especially when they are present in low abundance. Similar approaches based on MS fragmentation of different oligosaccharides have been used to distinguish between different sugar residues (Garozzo et al., 1990; Verardo et al., 2009), stereochemistry (Fang and Bendiak, 2007), linkage positions (Li and Her, 1998; Spengler et al., 1990), branching patterns (Cheng and Her, 2002; Daikoku et al., 2009) and anomeric configurations of the glycosidic bond (Mulroney et al., 1995; Yamagaki and Sato, 2009).

In general, plant species containing aromatic cyanogenic glucosides contain a single cyanogenic glucoside and in limited instances, the corresponding β(1 → 6) linked diglucoside. For example, prunasin and amygdalin have been reported to co-occur in the seeds of Rosaceaeous species (Dicenta et al., 2002; Miller et al., 2004; Vetter, 2000). Similarly dhurrin, has been reported to co-occur with its gentiobioside in *S. bicolor* (Poaceae; Selmar et al., 1996). Although gentiobiosides are the most common disaccharide associated with aromatic cyanogenic glucosides, other sugar moieties such as xylose and apiose have been reported in cyanogenic diglycosides in *Clerodendrum grayi* (Lamiaceae; Miller et al., 2006), *Xeranthemum cylindraceum* (Asteraceae; Schwind et al., 1990), and in *Davallia* species (Davalliaceae; Kofod and Eyjólfsson, 1969). In all the above instances, a β(1 → 6) linkage exists between the two sugar moieties of the diglycosides except for the aforementioned **5** isolated from *E. maculata* and *Perilla frutescens* (Aritomi et al., 1985; Syah and Ghisalberti, 1996). It is therefore unprecedented that *E. camphora* was found to produce four cyanogenic diglucosides with the glucose moieties coupled by β(1 → 6), β(1 → 2), β(1 → 3) and β(1 → 4) linkages.

The relative abundance of these diglucosides vary with plant and tissue age and with tissue type. A similar structural diversity of cyanogenic glucoside esters has been found in leaves of *Phyllagathis rotundiflora* (Melastomataceae) where seven different mono-, di-, tri- and tetra-galloylated forms of prunasin positioned at the

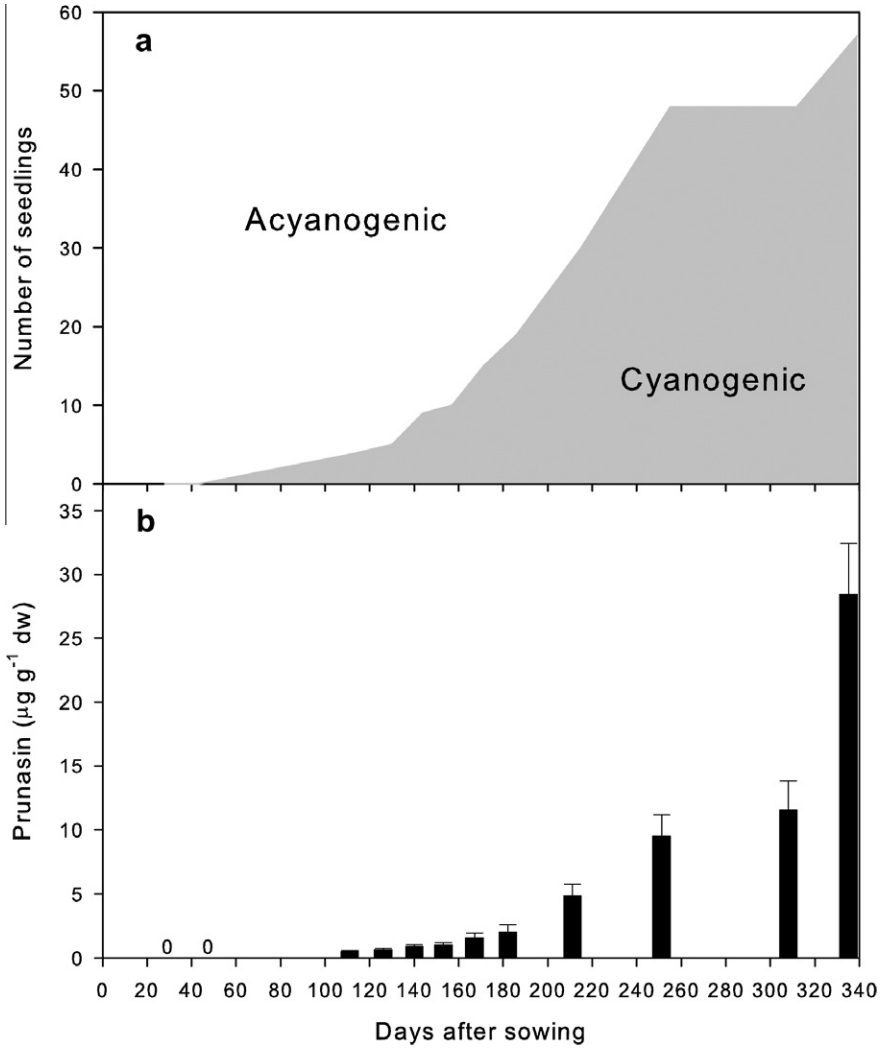

Fig. 4. The time dependent production of foliar prunasin in individually analyzed *Eucalyptus camphora* seedlings. (a) All 57 seedlings were initially incapable of synthesizing prunasin. Prunasin biosynthesis was detected at differing starting points but all seedlings produced prunasin 339 days after sowing. (b) Once each individual plant began to synthesize prunasin, its foliar concentration increased with time. Bars represent mean (±1 s.e.) of all cyanogenic seedlings at each harvest date.

C-2′, C-3′, C-4′ and C-6′ carbons were detected (Ling et al., 2002). Although the authors showed that these compounds were absent from root and stem tissues, no further tissue specificity or ontogenetic variation was examined.

3.2. Possible diversification of cyanogenic diglucoside function

The combined presence of the two monoglucosides prunasin and sambunigrin together with four cyanogenic diglucosides in adult *E. camphora* tissues may provide a measure of defense superior to that achieved in plants containing a single cyanogenic glucoside. The cleavage of the cyanogenic diglucosides may occur sequentially or simultaneously depending on whether the two sugar residues are removed in turn or as a disaccharide unit (Poulton, 1990; Selmar, 1993). If hydrolysis of the four different cyanogenic diglucosides present in *E. camphora* was to follow a simultaneous pathway, the diglucosides gentiobiose, cellobiose, sophorose and laminaribiose would be formed. Therefore, in addition to the toxic cyanide and deterrent benzaldehyde released, the diglucosides formed may serve as signals that elicit other strong plant defense responses (Field, 2009; Shibuya and Minami, 2001; Morrow and Lucas, 1986). For example, disaccharide formation may stimulate (1–3)-β-glucan synthase activity and callose production (see Morrow and Lucas, 1986), thus forming a protective physical barrier around the site of tissue damage (Stone and Clarke, 1992). Regardless of whether hydrolysis occurs sequentially or simultaneously, it is likely that *E. camphora* possess multiple β-glucosidases, each capable of acting upon a different glucosidic linkage type and potentially specific to each of the different cyanogenic diglucosides. High affinity towards particular disaccharide moieties has been observed for other plant β-glucosidases. For example, a β-primeverosidase from *Camellia sinensis* shows high selective activity towards a β-primeveroside moiety, will hydrolyze other disaccharides containing an α(1–6) or β(1–6) linkage to some degree, but does not hydrolyze α(1–4) or β(1–4) linked disaccharide moieties (Sakata et al., 2003). Similarly, a vicianin hydrolase from *Vicia angustifolia* hydrolyzes its native substrate, the cyanogenic glycoside vicianin with a β-vicianoside moiety, but not amygdalin with a β-gentbioside moiety (Ahn et al., 2007). Future work should focus on characterizing the enzyme(s) that hydrolyze the multiple cyanogenic glucosides in *E. camphora*.

Although it is likely that the mono-glucosides in *E. camphora* play a role in defense against herbivory, the cyanogenic diglucosides in this species may play additional roles related to nitrogen storage and transport, pollinator attraction and possibly even seedling germination. For example, it is known from studies on adult

Hevea brasiliensis trees that the cyanogenic diglucoside linustatin is translocated away from its site of synthesis in leaves, whereas the monoglucoside linamarin remains stored in leaves. Linustatin is translocated to inner bark of the trunk where it is endogenously catabolized and used for latex regeneration and rubber production (Kongsawadworakul et al., 2009). The diglucosides found in *E. camphora* may play a similar role in transport to organs such as developing leaves or flowers. Given the highest levels of diglucosides were found in flower buds and expanded leaves of adult *E. camphora* trees, it is tempting to suggest that the diglucosides are synthesized in the fully expanded leaves and transported to the developing flower buds. The much lower level of diglucosides and indeed total cyanogenic glucosides in immature fruits suggests nitrogen may have been remobilized from these compounds and possibly used in flower development or incorporated into pollen and nectar as pollinator cues. With respect to this latter possibility, the honey bee (*Apis mellifera*) has been shown to be able to detect and distinguish between different levels of amygdalin in floral parts of almonds (London-Shafir et al., 2003).

Finally, the cyanogenic diglucosides may also function as regulators of seed germination. For example, the cyanogenic diglucoside eucalyptosin A (**5**) described here in *E. camphora* tissues has previously been linked to germination inhibition in *E. maculata* (Richmond and Ghisalberti, 1994; Syah and Ghisalberti, 1996). *E. maculata* grows in arid regions throughout central Australia and chemical inhibitors in the fruit wall prevent germination until significant rainfall leaches them out, thereby enabling seed germination to occur (Chinnock, 2007; Richmond and Ghisalberti, 1994). In particular, **5** has been shown to be a component of the fruit wall and indeed when this diglucoside was extracted and applied directly to seeds, germination was completely inhibited (Syah and Ghisalberti, 1996). In a similar way, the cyanogenic diglucosides in *E. camphora* may also signal, delay or inhibit regulatory pathways such as seed germination.

3.3. Distribution of cyanogenic glucosides at different ontogenetic stages

Quantitative and qualitative variation in total cyanogenic glucoside abundance was observed throughout different ontogenetic stages in *E. camphora*. In particular, the onset of cyanogenic glucoside biosynthesis was highly variable with individuals "switching on" prunasin biosynthesis for the first time between 115 and 339 DAS (Fig. 4a). By 340 DAS, all half-sibling family members were cyanogenic, an observation consistent with the lack of acyanogenic plants in two previously screened adult populations (Neilson et al., 2006). This is the first time such ontogenetic polymorphism has been demonstrated, whereby genetically based variation in the timing of chemical defense initiation is apparent.

Once prunasin production was initiated, cyanogenic capacity increased through time with the biosynthesis of cyanogenic diglucosides initiated during the developmental stage between seedlings 141 DAS and saplings 1553 DAS (Figs. 3 and 4, Table 1). Total foliar cyanogenic glucoside concentration continually increased from seeding to sapling to adult in a similar manner to that observed for prunasin in *Eucalyptus polyanthemos* (Goodger et al., 2004), where maximum prunasin concentrations were also observed in reproductively mature adults. Nevertheless this ontogenetic strategy appears to differ from that of other cyanogenic eucalypts such as *Eucalyptus yarraensis*, where prunasin concentration reaches a maximum at 240 DAS (Goodger et al., 2007), and *Eucalyptus cladocalyx* where prunasin concentration is greatest at an even earlier stage (100 DAS; Goodger et al., 2006). Such variable ontogenetic strategies in cyanogenic eucalypts are likely to reflect a balance between growth and defense in environments with differing nutrient availability and herbivore pressures (Goodger et al., 2006). The availability of eucalypt species exhibiting different onset of cyanogenic glucoside production offers a unique model system to identify transcription factors involved in regulation of cyanogenic glucoside formation.

4. Concluding remarks

Adult *E. camphora* foliage and reproductive tissues contain two cyanogenic glucosides and four cyanogenic diglucosides. *E. camphora* displays strong ontogenetic and tissue-specific control over both the relative abundance of these compounds and total cyanogenic glucoside content. These attributes make *E. camphora* an excellent model species for studying the role of multiple cyanogenic glucosides in plants, to identify the UDP-glucosyltransferases responsible for diglucoside formation and to study the differential degradation of the cyanogenic diglucosides by β-glucosidases.

5. Experimental

5.1. General

LC-MS analyses were performed on an Agilent 1200 series LC system (Agilent, Santa Clara, USA) connected to a quadrupole-orthoganal time-of-flight (Q-TOF) mass spectrometer (Agilent 6520 system) and fitted with a Zorbax SB-Aq column (2.1 mm × 150 mm, 3.5 μm, 40 °C). Elution (0.5 mL min^{-1}) was carried out using a MeCN (0.1% formic acid) gradient from 10% to 20% over 5 min, increased to 80% over 3.5 min, followed by 1 min at 80%. ESI-MS was carried out in negative-ion mode (nebuliser pressure 40 psi, gas temperature 320 °C, capillary voltage 4000 V, fragmentor 150 V, skimmer 65 V and collision energy 25 V). The instrument was operated in the extended dynamic range mode and data collected in from 40–1700 amu with a scan rate of 2 scans s^{-1}.

GC–MS analysis was performed using a 7890A Agilent gas chromatograph coupled to a 5975C Agilent quadrupole mass spectrometer (Agilent, Santa Clara, USA) fitted with a VF-5MS column (30 m with 0.2 μm film thickness; Agilent) and an Integra guard column (10 m; SGE, Ringwood, Australia). Samples were dried *in vacuo* and derivatised for 120 min at 37 °C in 10 μL of 30 mg mL^{-1} methoxyamine hydrochloride in pyridine followed by treatment for 30 min at 37 °C with 20 μL of BSTFA and 2 μL of a retention time standard mixture (0.029% (v/v) *n*-dodecane, *n*-pentadecane, *n*-nonadecane, *n*-docosane, *n*-octacosane, *n*-dotriacontane, *n*-hexatriacontane dissolved in pyridine). Aliquots (1 μL) were injected onto the GC column using a hot needle technique at 250 °C and separated using a temperature program from 70 to 325 °C (7 °C min^{-1}, flow rate 0.8 mL min^{-1}). Authentic **3**, **5** and **6** were used as standards.

NMR spectra (^{1}H, cosydq, jres, noesy, tocsy) of **5** and **6** were recorded in methanol-*d*4 on a Bruker Avance 400 instrument (^{1}H NMR at 400 MHz, ^{13}C NMR at 100.6 MHz). ^{1}H shifts are relative to internal TMS; ^{13}C shifts are based on δ (C6′) = 62.6 ppm. All reported ^{13}C shifts were extracted from the 2D spectra hsqc or hmbc.

5.2. Plant material

E. camphora subsp. *humeana* L.A.S. Johnson & K.D. Hill (mountain swamp gum; henceforth referred to as *E. camphora*) is a small to medium-sized tree of southeastern Australia (Brooker and Kleinig, 2006). A population of adult trees was sampled from the Buxton region, Victoria, Australia (37°25′22.8″S, 145°42′32.4″E) and bulk samples of foliage (separated into expanding and expanded leaves), flower buds, and immature fruit (non-dehisced) harvested for cyanogenic glucoside compositional analysis in March, 2009. Seed was also collected at this time and sown directly into individual pots and placed in a glasshouse (see Goodger et al. (2007) for

germination and growth conditions). A bulk sample of fully expanded leaves was harvested from the seedlings 141 DAS as part of the compositional analysis. A previous cohort of 60 seedlings derived from the same population was germinated in the same glasshouse in February 2005. A single leaf was harvested from each seedling every few weeks until 339 DAS as part of the ontogenetic study of cyanogenic capacity. At each harvest, plant height was determined as a measure of biomass. Twenty-four of these plants were relocated to Alberton, Victoria (38°36′45″S, 146°39′58″E) on 22 October, 2006 and left to grow under natural environmental conditions. A bulk sample of fully expanded leaves was harvested from these reproductively immature saplings as part of the compositional analysis in May, 2009 (1553 DAS). Adult *E. maculata* subsp. *brevifolia* (Benth.) Chinnock (spotted emu bush or native fuchsia; henceforth referred to as *E. maculata*) plants possessing fruit were purchased from Wail Nursery (Wail, Victoria).

5.3. Structural elucidation of **5**, **6** and **7**

The bulk samples of leaf and reproductive tissues were homogenized in liquid N_2 using a mortar and pestle. Freshly homogenized samples (20 g) of *E. camphora* leaves, flower buds and fruits as well as *E. maculata* fruits were defatted using petroleum ether (solvent:tissue, 10:1 v/w, four extractions), and then twice extracted with cold MeOH. The MeOH filtrate was concentrated under N_2 and an equivalent volume of $CHCl_3$ added with sufficient water to enable phase separation. The aqueous phase was collected, concentrated to dryness *in vacuo*, and the residual material re-suspended in water, filtered and fractionated using an Alltech Maxi-Clean C_{18} SPE cartridge (900 mg; Deerfield, USA) using gradient elution (1 mL min^{-1}, 0–100% MeOH–H_20). The fractions obtained were concentrated *in vacuo* and cyanogenic glucoside derived HCN release was monitored following addition of a commercial preparation of emulsin, the cyanogenic glucoside cleaving β-glucosidase from almond (*Prunus amygdalis*; β-D-glucoside glucohydrolase; EC 3.2.1.21, Sigma–Aldrich). Cyanogenic glucoside-containing fractions were analyzed by HPLC using a Phenomenex Luna C_{18} (2) column (250 mm × 10 mm, 5 μm particles; Torrance, USA) and eluted with 20% MeCN (2 mL min^{-1}).

Amygdalin standard was purchased from Sigma–Aldrich (Sydney, Australia). Neoamygdalin was synthesized from the amygdalin standard (100 mg) by addition of 10 mL of 5 mM ammonia and incubation (room temperature, 2 h). During this time, equilibrium between amygdalin and neoamygdalin was established (Fischer, 1885; Koo et al., 2005).

5.3.1. (*R*)-mandelonitrile β-sophoroside, eucalyptosin A (**5**)

Cyanogenic diglucoside **5** was compared to a standard isolated from mature fruits of *E. maculata* (Syah and Ghisalberti, 1996). Structural characterization: UV λ_{max} (MeCN) 208 nm; ^{1}H NMR (400 MHz, CD_3OD) ^{1}H NMR δ: 7.65 (H-2), 7.45 (H-3), 5.91 (H-7), 4.61 (J = 7.8 Hz, H-1″), 4.51 (J = 7.1 Hz, H-1′), 3.61 (H-2′), 3.53 (H-3′), 3.21 (H-2″). ^{13}C NMR (100 MHz, CD_3OD) δ: 135.1 (C-1), 128.6 (C-2), 130.2 (C-3), 69.1 (C-7), 119.5 (C-8), 101.5 (C-1′), 82.7 (C-2′), 77.9 (C-3′), 62.6 (C-6′), 105.3 (C-1″). MS (ESI^-) m/z: 502.1525 (calcd. 502.1555 for $C_{20}H_{27}N_1O_{11}$ $C_1H_1O_2$). MS^2 fragmentation of $[M+C_1H_1O_2]^-$ (rel. int.): 89.0245 (100), 161.0455 (87), 59.0142 (65), 101.0243 (55), 71.014 (47), 456.1512 (45), 119.0347 (43), 113.0241 (38) and 131.0368 (30).

5.3.2. Mandelonitrile β-cellobioside 2-(S,R)-[(4-O-β-D-glucopyranosyl) -β-D-glucopyranosyloxy]-2-phenylacetonitrile, eucalyptosin B (**6**)

Cyanogenic diglucoside **6** was chemically synthesized as an epimeric mixture (in the *ratio* of 2:1) in six steps using cellobiose and benzaldehyde as starting materials and proceeding with β-D-glucopyranosyl-(1 → 4)-D-glucopyranose (cellobiose), 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-(1 → 4)-1,2,3,6-tetra-*O*-acetyl-D-glucopyranose, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-D-glucopyranose, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-D-glucopyranosyl fluoride, 2-phenyl-2-trimethylsilyoxyacetonitrile and 2-(*S*,*R*)-[(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-(1 → 4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyloxy]-2-phenylacetonitrile as intermediates. Detailed chemical synthesis and experimental data are to be published elsewhere (Motawia et al., unpublished data). Structural characterization: UV λ_{max} (MeCN) 208 nm; ^{1}H NMR (400 MHz, CD_3OD) δ: 7.62–7.33 (5H, m, H-arom), 6.02, 5.90 (1H, 2s, C*H*CN, epimers), 4.74 (J = 8.0 Hz), 4.43 (J = 8.0 Hz), 4.42 (J = 8.0 Hz), 4.34 (J = 7.6 Hz) (4d, 2H, H-1$_\beta$ & H-1′$_\beta$, epimers); ^{13}C NMR (100 MHz, CD_3OD) δ: 135.2, 134.9, 131.1, 130.8, 130.2, 130.2, 130.0, 130.0, 129.0, 129.0, 128.8, 128.8 (C-arom), 119.4, 118.5 (*C*N, epimers), 104.6, 104.6 (C-1, epimers), 102.2, 101.9 (C-1′, epimers), 80.5, 80.5,78.2, 78.2, 77.9, 77.9, 77.9, 77.1,76.9, 76.3, 76.3, 74.9, 74.9, 74.5, 71.4, 71.4 (C-2, C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′, cellobiose), 68.8, 68.7 (*C*HCN, epimers), 62.5, 62.5, 61.8, 61.6 (2C-6, 2C-6′, cellobiose epimers). MS (ESI^-) m/z: 502.1533 (calcd. 502.1555 for $C_{20}H_{27}N_1O_{11}$ $C_1H_1O_2$). MS^2 fragmentation of $[M+C_1H_1O_2]^-$ (rel. int.): 161.0453 (100), 89.0242 (58), 101.0243 (46), 113.0241 (34), 59.0143 (29), 71.0142 (29), 456.1522 (29) and 119.0356 (25).

5.3.3. Mandelonitrile β-laminaribioside, eucalyptosin C (**7**)

Cyanogenic diglucoside **7** was elucidated based on LC-MS data and MS^2 fragmentation patterns and fragment abundance. UV λ_{max} (MeCN) 208 nm; MS (ESI^-) m/z: 502.1506 (calcd. 502.1555 for $C_{20}H_{27}N_1O_{11}$ $C_1H_1O_2$). MS^2 fragmentation of $[M+C_1H_1O_2]^-$ (rel. int.): 161.0448 (100), 323.0974 (85), 113.0237 (79), 101.0246 (76), 89.0243 (66), 59.0141 (59), 71.0131 (33) and 119.0366 (30).

5.4. Quantitative determination of HCN potential and individual cyanogenic glucosides

5.4.1. Individual cyanogenic glucosides

Quantification of individual cyanogenic glucosides present in different *E. camphora* tissues was performed by LC-MS. Homogenized plant material (25 mg) was boiled in 80% MeOH (1 mL, 5 min), vortexed (15 s) and centrifuged (10 min, 5000*g*). To remove hydrophobic components present in the sample, the supernatant was diluted to a final concentration of 50% MeOH and one volume of $CHCl_3$ added to enable phase separation. The MeOH phase obtained following vortexing (15 s) and centrifugation (5 min, 5000*g*) was diluted 1:20 and filtered (0.22 μm low-binding Durapore membrane). Quantification was performed by comparing the extracted ion chromatogram of the different cyanogenic glucosides to an external standard series of authentic prunasin (Sigma, St. Louis, USA) and amygdalin of known concentrations (2–200 ng). Duplicate samples were extracted and analyzed for each tissue type and the resultant values are the means of the duplicates.

5.4.2. Total cyanogenic capacity

Freeze-dried plant tissue (40 mg) was placed in a glass vial with a 0.5 mL tube containing 100 μL 1 M NaOH. The vial tissue was hydrated with 1 mL of 0.1 mM citrate buffer (pH 5.5) and the vial immediately sealed. Following incubation (18 h, 37 °C), cyanide trapped in the NaOH solution was determined using a miniaturized colorimetric (590 nm) microtiterplate based cyanide assay (Brinker and Seigler, 1989; Goodger et al., 2002). To quantify the amount of cyanide each plant tissue can produce naturally, i.e. cyanogenic capacity, no exogenous β-glucosidase enzyme was added.

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References

Ahn, Y.O., Saino, H., Mizutani, M., Shimizu, B., Sakata, K., 2007. Vicianin hydrolase is a novel cyanogenic β-glycosidase specific to β-vicianoside (6-*O*-α-L-arabinopyranosyl-β-D-glucopyranoside) in seeds of *Vicia angustifolia*. Plant and Cell Physiology 48, 938–947.

Aritomi, M., Kumori, T., Kawasaki, T., 1985. Chemical studies on the constituents of edible plants. 4. Cyanogenic glycosides in leaves of *Perilla frutescens* var. *acuta*. Phytochemistry 24, 2438–2439.

Bjarnholt, N., Møller, B.L., 2008. Hydroxynitrile glucosides. Phytochemistry 69, 1947–1961.

Bohlmann, J., Keeling, C.I., 2008. Terpenoid biomaterials. The Plant Journal 54, 656–669.

Brinker, A.M., Seigler, D.S., 1989. Methods for the detection and quantitative determination of cyanide in plant materials. Phytochemical Bulletin 21, 24–31.

Brooker, M.I.H., Kleinig, D.A., 2006. Field Guide to Eucalypts. Bloomings Books, Melbourne.

Busk, P.K., Møller, B.L., 2002. Dhurrin synthesis in sorghum is regulated at the transcriptional level and induced by nitrogen fertilization in older plants. Plant Physiology 129, 1222–1231.

Cheng, H.L., Her, G.R., 2002. Determination of linkages of linear and branched oligosaccharides using closed-ring chromophore labeling and negative ion trap mass spectrometry. Journal of the American Society for Mass Spectrometry 13, 1322–1330.

Chinnock, R.J., 2007. *Eremophila* and Allied Genera: A Monograph of the Plants of Myoporaceae. Rosenberg publishing Pty Ltd, New South Wales.

Cmelík, R., Chmelík, J., 2010. Structural anallysis and differentiation of reducing and nonreducing neutral model starch oligosaccharides by negative-ion electrospray ionization ion-trap mass spectrometry. International Journal of Mass Spectrometry 2010, 33–40.

Daikoku, S., Widmalm, G., Kanie, O., 2009. Analysis of a series of isomeric oligosaccharides by energy-resolved mass spectrometry: a challenge on homobranched trisaccharides. Rapid Communications in Mass Spectrometry 23, 3713–3719.

Dicenta, F., Martinez-Gomez, P., Grane, N., Martin, M.L., Leon, A., Canovas, J.A., Berenguer, V., 2002. Relationship between cyanogenic compounds in kernels, leaves, and roots of sweet and bitter kernelled almonds. Journal of Agricultural and Food Chemistry 50, 2149–2152.

Domon, B., Costello, C.E., 1988. A systematic nomenclature for carbohydrate fragmentations in FAB-MS MS spectra of glycoconjugates. Glycoconjugate Journal 5, 397–409.

Fang, T.T., Bendiak, B., 2007. The stereochemical dependence of unimolecular dissociation of monosaccharide-glycolaldehyde anions in the gas phase: a basis for assignment of the stereochemistry and anomeric configuration of monosaccharides in oligosaccharides by mass spectrometry via a key discriminatory product ion of disaccharide fragmentation, *m*/*z* 221. Journal of the American Chemical Society 129, 9721–9736.

Field, R.A., 2009. Oligosaccharide signalling molecules. In: Osbourn, A.E., Lanzotti, V. (Eds.), Plant-derived Natural Products. Springer, New York.

Fischer, E., 1885. Ueber ein neues dem Amygdalin ähnliches Glucosid. Berichte der Deutschen Chemischen Gesellschaft 28, 1508–1511.

Fleming, F.F., 1999. Nitrile-containing natural products. Natural Product Reports 16, 597–606.

Garozzo, D., Giuffrida, M., Impallomeni, G., Ballistreri, A., Montaudo, G., 1990. Determination of linkage position and identification of the reducing end in linear oligosaccharides by negative-ion fast atom bombardment mass-spectrometry. Analytical Chemistry 62, 279–286.

Goodger, J.Q.D., Ades, P.K., Woodrow, I.E., 2004. Cyanogenesis in *Eucalyptus polyanthemos* seedlings: heritability, ontogeny and effect of soil nitrogen. Tree Physiology 24, 681–688.

Goodger, J.Q.D., Capon, R.J., Woodrow, I.E., 2002. Cyanogenic polymorphism in *Eucalyptus polyanthemos* Schauer subsp *vestita* L. Johnson and K. Hill (Myrtaceae). Biochemical Systematics and Ecology 30, 617–630.

Goodger, J.Q.D., Choo, T.Y.S., Woodrow, I.E., 2007. Ontogenetic and temporal trajectories of chemical defence in a cyanogenic eucalypt. Oecologia 153, 799–808.

Goodger, J.Q.D., Gleadow, R.M., Woodrow, I.E., 2006. Growth cost and ontogenetic expression patterns of defence in cyanogenic *Eucalyptus* spp.. Trees-Structure and Function 20, 757–765.

Jenrich, R., Trompetter, I., Bak, S., Olsen, C.E., Møller, B.L., Piotrowski, M., 2007. Evolution of heteromeric nitrilase complexes in Poaceae with new functions in nitrile metabolism. Proceedings of the National Academy of Sciences of the United States of America 104, 18848–18853.

Jones, P.R., Andersen, M.D., Nielsen, J.S., Hoj, P.B., Møller, B.L., 2000. The biosynthesis, degradation, transport and possible function of cyanogenic glucosides. In: Romeo, J.T., Ibrahim, R., Varin, L., DeLuca, V. (Eds.), Evolution of Metabolic Pathways, vol. 34, pp. 191–247.

Jørgensen, K., Bak, S., Busk, P.K., Sorensen, C., Olsen, C.E., Puonti-Kaerlas, J., Møller, B.L., 2005a. Cassava plants with a depleted cyanogenic glucoside content in leaves and tubers. Distribution of cyanogenic glucosides, their site of synthesis and transport, and blockage of the biosynthesis by RNA interference technology. Plant Physiology 139, 363–374.

Jørgensen, K., Rasmussen, A.V., Morant, M., Nielsen, A.H., Bjarnholt, N., Zagrobelny, M., Bak, S., Møller, B.L., 2005b. Metabolon formation and metabolic channeling in the biosynthesis of plant natural products. Current Opinion in Plant Biology 8, 280–291.

Kofod, H., Eyjólfsson, R., 1969. Cyanogenesis in species of fern genera *Cystopteris* and *Davallia*. Phytochemistry 8, 1509–1511.

Kongsawadworakul, P., Viboonjun, U., Romruensukharom, P., Chantuma, P., Ruderman, S., Chrestin, H., 2009. The leaf, inner bark and latex cyanide potential of *Hevea brasiliensis*: evidence for involvement of cyanogenic glucosides in rubber yield. Phytochemistry 70, 730–739.

Koo, J.Y., Hwang, E.Y., Cho, S., Lee, J.H., Lee, Y.M., Hong, S.P., 2005. Quantitative determination of amygdalin epimers from Armeniacae semen by liquid chromatography. Journal of Chromatography B – Analytical Technologies in the Biomedical and Life Sciences 814, 69–73.

Li, D.T., Her, G.R., 1998. Structural analysis of chromophore-labeled disaccharides and oligosaccharides by electrospray ionization mass spectrometry and high-performance liquid chromatography/electrospray ionization mass spectrometry. Journal of Mass Spectrometry 33, 644–652.

Ling, S.K., Tanaka, T., Kouno, I., 2002. New cyanogenic and alkyl glycoside constituents from *Phyllagathis rotundifolia*. Journal of Natural Products 65, 131–135.

London-Shafir, I., Shafir, S., Eisikowitch, D., 2003. Amygdalin in almond nectar and pollen – facts and possible roles. Plant Systematics and Evolution 238, 87–95.

Miller, R.E., Gleadow, R.M., Woodrow, I.E., 2004. Cyanogenesis in tropical *Prunus turneriana*: characterisation, variation and response to low light. Functional Plant Biology 31, 491–503.

Miller, R.E., McConville, M.J., Woodrow, I.E., 2006. Cyanogenic glycosides from the rare Australian endemic rainforest tree *Clerodendrum grayi* (Lamiaceae). Phytochemistry 67, 43–51.

Møller, B.L., 2010. Functional diversifications of cyanogenic glucosides. Current Opinion in Plant Biology 13, 338–347.

Møller, B.L., Conn, E.E., 1980. The biosynthesis of cyanogenic glucosides in higher plants: channelling of intermediates in dhurrin biosynthesis by a microsomal system from *Sorghum bicolor* (L.) Moench. Journal of Biological Chemistry 255, 3049–3056.

Morrow, D.L., Lucas, W.J., 1986. (1–3)-β-D-Glucan synthase from sugar beet. I. Isolation and solubilization. Plant Physiology 81, 171–176.

Mulroney, B., Traeger, J.C., Stone, B.A., 1995. Determinatoin of both linkage position and anomeric configuration in underivatised glucopyranosyl disaccharides by electrospray mass-spectrometry. Journal of Mass Spectrometry 30, 1277–1283.

Nahrstedt, A., 1975. Isomerization of amygdalin and its homologues. Archiv Der Pharmazie 308, 903–910.

Neilson, E.H., Goodger, J.Q.D., Woodrow, I.E., 2006. Novel aspects of cyanogenesis in *Eucalyptus camphora* subsp *humeana*. Functional Plant Biology 33, 487–496.

Nielsen, K.A., Tattersall, D.B., Jones, P.R., Møller, B.L., 2008. Metabolon formation in dhurrin biosynthesis. Phytochemistry 69, 88–98.

Poulton, J.E., 1990. Cyanogenesis in plants. Plant Physiology 94, 401–405.

Richmond, G.S., Ghisalberti, E.L., 1994. Seed dormancy and germination mechanisms in *Eremophila* (Myoporaceae). Australian Journal of Botany 42, 705–715.

Sakata, K., Mizutani, M., Ma, S.J., Hiratake, J., 2003. Diglycoside-specific glycosidases. Recognition of Carbohydrates in Biological Systems, Part B: Specific Applications 363, 444–459.

Sánchez-Pérez, R., Jørgensen, K., Olsen, C.E., Dicenta, F., Møller, B.L., 2008. Bitterness in almonds. Plant Physiology 146, 1040–1052.

Schwind, P., Wray, V., Nahrstedt, A., 1990. Structure elucidatoin of an acylated cyanogenic triglycoside, and further cyanogenic constituents from *Xeranthemum cylindraceum*. Phytochemistry 29, 1903–1911.

Selmar, D., 1993. Transport of cyanogenic glucosides – linustatin uptake by *Hevea* cotyledons. Planta 191, 191–199.

Selmar, D., Irandoost, Z., Wray, V., 1996. Dhurrin-6′-glucoside, a cyanogenic diglucoside from *Sorghum bicolor*. Phytochemistry 43, 569–572.

Selmar, D., Lieberei, R., Biehl, B., 1988. Mobilization and utilization of cyanogenic glycosides – the linustatin pathway. Plant Physiology 86, 711–716.

Shibuya, N., Minami, E., 2001. Oligosaccharide signalling for defence responses in plant. Physiological and Molecular Plant Pathology 59, 223–233.

Spengler, B., Dolce, J.W., Cotter, R.J., 1990. Infrared-laser desorption mass-spectrometry of oligosaccharides – fragmentation mechanisms and isomer analysis. Analytical Chemistry 62, 1731–1737.

Stone, B.A., Clarke, A.E., 1992. Chemistry and Biology of (1–3)-β-D-Glucans. La Trobe University Press, Victoria, Australia.

Swain, E., Poulton, J.E., 1994. Utilization of amygdalin during seedling development of *Prunus serotina*. Plant Physiology 106, 437–445.

Syah, Y.M., Ghisalberti, E.L., 1996. Biologically active cyanogenetic, iridoid and lignan glycosides from *Eremophila maculata*. Fitoterapia 67, 447–451.
Verardo, G., Duse, I., Callea, A., 2009. Analysis of underivatized oligosaccharides by liquid chromatography/electrospray ionization tandem mass spectrometry with post-column addition of formic acid. Rapid Communications in Mass Spectrometry 23, 1607–1618.
Vetter, J., 2000. Plant cyanogenic glycosides. Toxicon 38, 11–36.
Winkel, B.S.J., 2004. Metabolic channeling in plants. Annual Review of Plant Biology 55, 85–107.
Wong, A.W., Cancilla, M.T., Voss, L.R., Lebrilla, C.B., 1999. Anion dopant for oligosaccharides in matrix-assisted laser desorption/ionization mass spectrometry. Analytical Chemistry 71, 205–211.
Yamagaki, T., Sato, A., 2009. Isomeric oligosaccharides analyses using negative-ion eectrospray ionization ion mobility spectrometry combined with collision-induced dissociation MS/MS. Analytical Sciences 25, 985–988.
Zagrobelny, M., Bak, S., Moller, B.L., 2008. Cyanogenesis in plants and arthropods. Phytochemistry 69, 1457–1468.