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Diversification of an ancient theme: Hydroxynitrile glucosides

Nanna Bjarnholt [a], Fred Rook [a], Mohammed Saddik Motawia [a], Claus Cornett [b], Charlotte Jørgensen [a], Carl Erik Olsen [a,c], Jerzy W. Jaroszewski [d], Søren Bak [a], Birger Lindberg Møller [a,*]

[a] *Plant Biochemistry Laboratory and Center for Molecular Plant Physiology (PlaCe), Department of Plant Biology, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Copenhagen, Denmark*
[b] *Department of Pharmaceutics and Analytical Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark*
[c] *Department of Natural Sciences, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark*
[d] *Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark*

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Abstract

Many plants produce cyanogenic glucosides as part of their chemical defense. They are α-hydroxynitrile glucosides, which release toxic hydrogen cyanide (HCN) upon cleavage by endogenous plant β-glucosidases. In addition to cyanogenic glucosides, several plant species produce β- and γ-hydroxynitrile glucosides. These do not release HCN upon hydrolysis by β-glucosidases and little is known about their biosynthesis and biological significance. We have isolated three β-hydroxynitrile glucosides, namely (2*Z*)-2-(β-D-glucopyranosyloxy)but-2-enenitrile and (2*R*,3*R*)- and (2*R*,3*S*)-2-methyl-3-(β-D-glucopyranosyloxy)butanenitrile, from leaves of *Ribes uva-crispa*. These compounds have not been identified previously. We show that in several species of the genera *Ribes*, *Rhodiola* and *Lotus*, these β-hydroxynitrile glucosides co-occur with the L-isoleucine-derived hydroxynitrile glucosides, lotaustralin (α-hydroxynitrile glucoside), rhodiocyanosides A (γ-hydroxynitrile glucoside) and D (β-hydroxynitrile glucoside) and in some cases with sarmentosin (a hydroxylated rhodiocyanoside A). Radiolabelling experiments demonstrated that the hydroxynitrile glucosides in *R. uva-crispa* and *Hordeum vulgare* are derived from L-isoleucine and L-leucine, respectively. Metabolite profiling of the natural variation in the content of cyanogenic glucosides and β- and γ-hydroxynitrile glucosides in wild accessions of *Lotus japonicus* in combination with genetic crosses and analyses of the metabolite profile of the F2 population provided evidence that a single recessive genetic trait is most likely responsible for the presence or absence of β- and γ-hydroxynitrile glucosides in *L. japonicus*. Our findings strongly support the notion that the β- and γ-hydroxynitrile glucosides are produced by diversification of the cyanogenic glucoside biosynthetic pathway at the level of the nitrile intermediate.

Keywords: *Ribes*; *Lotus*; *Rhodiola*; Grossulariaceae; Crassulaceae; Fabaceae; Poaceae; Cyanogenic glucosides; Hydroxynitrile glucosides

1. Introduction

Cyanogenic glucosides are β-glucosides of α-hydroxynitriles (Conn, 1981) produced by many plant species. In 1999, approximately 60 naturally occurring cyanogenic glucosides and derivatives, such as disaccharides, had been identified (reviewed by Lechtenberg and Nahrstedt (1999) and Møller and Seigler (1999)) and a few have been added since (Jaroszewski et al., 2002; Nakamura et al., 2007). Except for one (apparently derived from nicotinic acid), cyanogenic glucosides are derived from the amino acids L-valine, L-isoleucine, L-leucine, L-phenylalanine, L-tyrosine or the non-protein amino acid L-2-(2′-cyclopentenyl)glycine (Bak et al., 2006; Lechtenberg and Nahrstedt, 1999; Møller and Seigler, 1999). Plants usually produce cyanogenic glucosides from a single amino acid precursor but cyanogenic glucosides derived from valine and isoleucine commonly

* Corresponding author. Tel.: +45 3528 3352; fax: +45 3528 3333.
E-mail address: blm@life.ku.dk (B.L. Møller).

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co-occur, and they in turn co-occur with those derived from 2-(2′-cyclopentenyl)glycine (Jaroszewski et al., 1988, 2002; Olafsdottir et al., 1989). Cyanogenic glucosides are stored in the vacuole of the plant cell. Upon disruption of the plant tissue e.g. by chewing insects, they are brought in contact with specific β-glucosidases which in the intact cell are localized in the chloroplast or apoplast (Gruhnert et al., 1994; Kakes, 1985; Saunders and Conn, 1978; Thayer and Conn, 1981). This results in hydrolysis of the cyanogenic glucosides and concomitant release of toxic hydrogen cyanide (HCN) and a ketone or aldehyde (Fig. 1). This binary system offers plants an immediate chemical defense response to herbivores and pathogens that cause tissue damage. Accordingly, the cyanogenic glucosides are categorized as phytoanticipins (Bak et al., 2006).

The ability to release toxic HCN from the cyanogenic glucosides relies on the inherent instability of the α-hydroxynitriles. The release is accelerated by endogenous plant α-hydroxynitrile lyases (Fig. 1). Several cyanogenic plants also produce glucosides of β- or γ-hydroxynitriles, which do not release HCN upon hydrolysis (Fig. 2). In *Lotus japonicus* (Regel) K. Larsen (Fabaceae) (Forslund et al., 2004) and species of *Rhodiola* (Crassulaceae) (Fan et al., 2001; Nakamura et al., 2007; Yoshikawa et al., 1996, 1997; Yousef et al., 2006) the isoleucine-derived cyanogenic glucoside lotaustralin (**9a**) and in one case also the hydroxylated cyanogenic glucoside, sachaloside V (**9c**), co-occur with the unsaturated β- and γ-hydroxynitrile glucosides rhodiocyanosides D (**9d**) and A (**9e**) (respectively (2*E*)-2-[(β-D-glucopyranosyloxy)methyl]-2-butene nitrile and (2*Z*)-2-[(β-D-glucopyranosyloxy)methyl]-2-butene nitrile) (Fig. 2). In *Hordeum vulgare* L. (barley, Poaceae) (Nielsen et al., 2002; Pourmohseni et al., 1993) and members of the Rosaceae (Lechtenberg et al., 1996) the leucine-derived cyanogenic glucoside epiheterodendrin (**10b**) co-occurs with the β-hydroxynitrile glucoside epidermin (**10d**), the γ-hydroxynitrile glucoside dihydroosmaronin (**10g**) and with unsaturated and/or hydroxylated analogs of these compounds (**10c**, **e** and **f**). The biological role of these β- and γ-hydroxynitrile glucosides in plants is virtually unexplored. Arthropods which contain sarmentosin (**9f**) or sutherlandin (**10f** (or its *E*-isomer)) were reported to be strongly deterrent against their predators (Braekman et al., 1982; Nishida et al., 1994). This may reflect a general property of the compounds also utilized by plants for defense against specialist plant pathogens and herbivores that have evolved to circumvent cyanogenic defense (Engler et al., 2000; Gleadow and Woodrow, 2002; Nielsen et al., 2006; Nishida et al., 1994; Zagrobelny et al., 2004, 2007a,b). The rhodiocyanosides and the related **9f** were suggested to have effects in treatment of allergy (Yoshikawa et al., 1996, 1997), chronic viral hepatitis and liver cancer (Zhu et al., 1996). The non-cyanogenic β- and γ-hydroxynitrile glucosides provide an opportunity to study evolutionary recruitment of known enzymes with new catalytic functions to enable the formation of new natural products from existing biosynthetic pathways. Furthermore, β- and γ-hydroxynitrile glucosides may find uses in terms of crop protection and biomedicine. Knowledge of the biosynthesis of β- and γ-hydroxynitrile glucosides is lacking, but studies in *L. japonicus* have shown that isoleucine is the common precursor of **9a**, **9d** and **9e** (Forslund et al., 2004) and that the isoleucine-derived **2** is most likely a common intermediate in the biosynthesis of the three compounds (Morant et al., 2007).

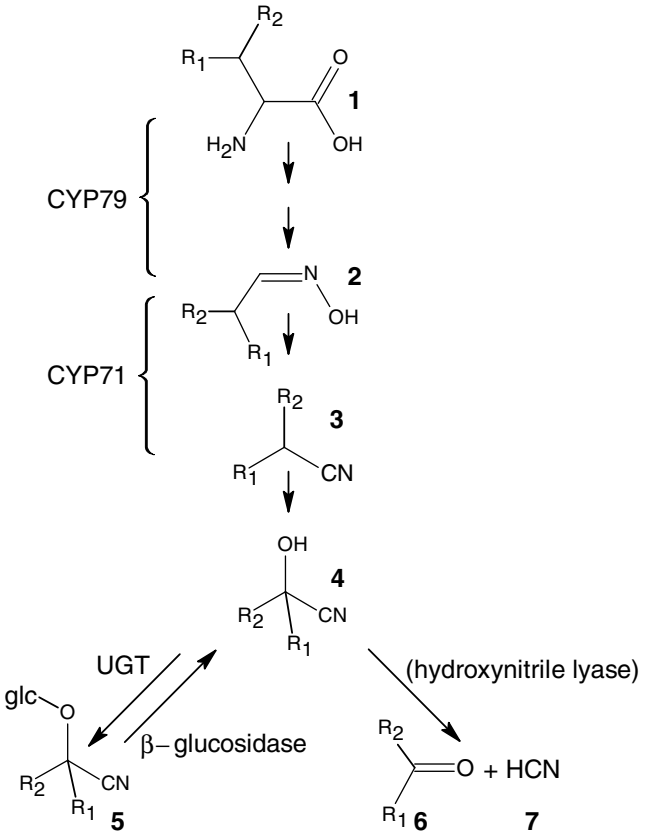

Fig. 1. Biosynthesis and bioactivation of cyanogenic glucosides. Biosynthesis: a multifunctional cytochrome P450, CYP79, converts the precursor amino acid (1) into the corresponding oxime (2) (Sibbesen et al., 1994, 1995). Subsequently, CYP71 catalyzes a multistep reaction, converting 2 to into a nitrile (3) and into an α-hydroxynitrile (cyanohydrin, 4) (Bak et al., 1998; Kahn et al., 1997), which is ultimately glucosylated by a UDP-glucosyl transferase (UGT) (Jones et al., 1999; Thorsoe et al., 2005). Bioactivation: upon tissue disruption the cyanogenic glucosides are hydrolyzed by specific β-glucosidases and the unstable α-hydroxynitriles (4) dissociate spontaneously or mediated by hydroxynitrile lyases to release ketone or aldehyde (6) and HCN (7).

In this paper we report the isolation and identification of three previously unknown β-hydroxynitrile glucosides from *Ribes uva-crispa* L. (gooseberry, Grossulariaceae) and show that they generally co-occur with known isoleucine-derived hydroxynitrile glucosides. Furthermore, we provide evidence that the biosynthetic pathways producing isoleucine and leucine-derived β- and γ-hydroxynitrile glucosides are most likely to be derived from the corresponding biosynthetic pathways of cyanogenic glucosides.

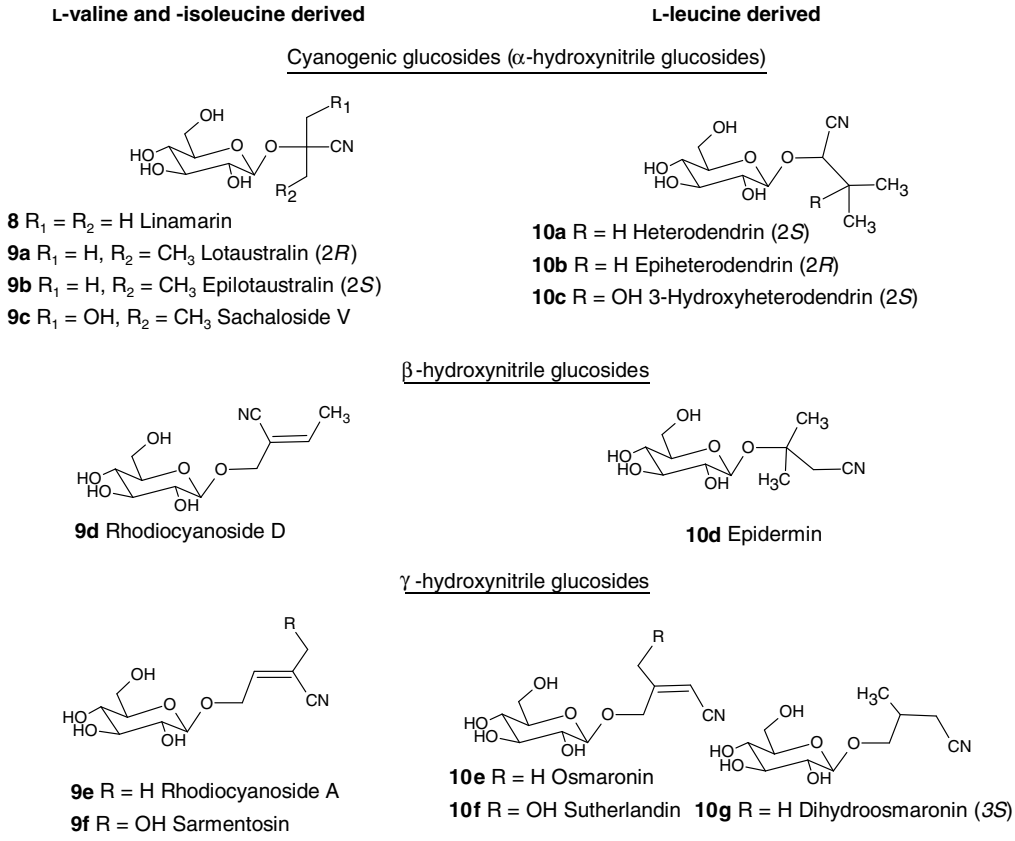

Fig. 2. Selected structures of aliphatic hydroxynitrile glucosides. The stereochemistry of **9c** is not resolved (Nakamura et al., 2007).

2. Results and discussion

Patterns of co-occurrence of isoleucine-derived α-, β- and γ-hydroxynitrile glucosides were investigated by thorough screening of plant species belonging to the genera which had previously been reported to contain rhodiocyanosides (*Rhodiola* (Fan et al., 1999, 2001; Ohsugi et al., 1999; Yoshikawa et al., 1996, 1997; Yousef et al., 2006), *Lotus* (Forslund et al., 2004) and *Jatropha* (Euphorbiaceae) (van den Berg et al., 1995)). The study also included plants of the genus *Ribes* from which the hydroxynitrile of **9d** has previously been isolated along with diastereomeric 3-hydroxy-2-methylbutanenitriles of unknown stereochemistry (Nishimura et al., 1987).

2.1. Identification of hydroxynitrile glucosides

Compounds **9a, 9d** and **9e** were isolated by preparative LC-MS from *L. japonicus* and used as authentic standards. The structures were verified by ^{1}H and ^{13}C NMR spectroscopy (data not shown) (Akgul et al., 2004; Seigler and Brinker, 1993; Yoshikawa et al., 1996, 1997). The saturated **9a** has no UV-absorption above 200 nm while **9d** and **9e** display absorption around 210 nm due to the double bond conjugation with the nitrile triple bond. The presence of sodium ions in electro-spray ion trap MS analysis facilitates formation of a fragment ion of $m/z = 185$ characteristic for glucosides ($[glc + Na–H_2O]^+$). The fragment $[M + Na–HCN]^+$ ($m/z = M + Na-27$) is diagnostic of cyanogenic glucosides (Franks et al., 2005; Hansen et al., 2003; Thorsoe et al., 2005) but the loss of HCN does not occur from β- and γ-hydroxynitrile glucosides (our unpublished results). The compounds **9a** and its epimer **9b** could not be separated in the LC-MS analysis and therefore the compound(s) from the *R. uva-crispa* extract leading to the corresponding peak of m/z 284 ($M + Na^+$) were also collected and subjected to ^{1}H NMR analysis. The analysis revealed that the collected fraction contained **9a** and **9b** in a ratio of 4:1. Along with **9a/b** the *R. uva-crispa* leaves contained **9d** and **9e**, and in addition two compounds with LC-MS and UV spectral characteristics corresponding to hypothetical non-cyanogenic isomers of **9a/b**, one with characteristics corresponding to a **9d/e** isomer and finally one matching a hydroxylated **9d/e** isomer (Fig. 3). The four unknown compounds were isolated from *R. uva-crispa* leaves by preparative LC-MS and the structures determined by NMR. The remaining plant extracts were analyzed by LC-MS and the presence of all the isoleucine-derived compounds was confirmed by m/z-values, retention times, UV-spectra and fragmentation in ion trap MS. The epimeric composition of **9a/b** was only established for *L. japonicus* and *R. uva-crispa*.

Structures of the isolated compounds were determined using a capillary NMR probe, which enables analysis of microgram amounts of material (Schlotterbeck et al., 2002). The compound of $t_R = 4.0$ min (Fig. 3) was con-

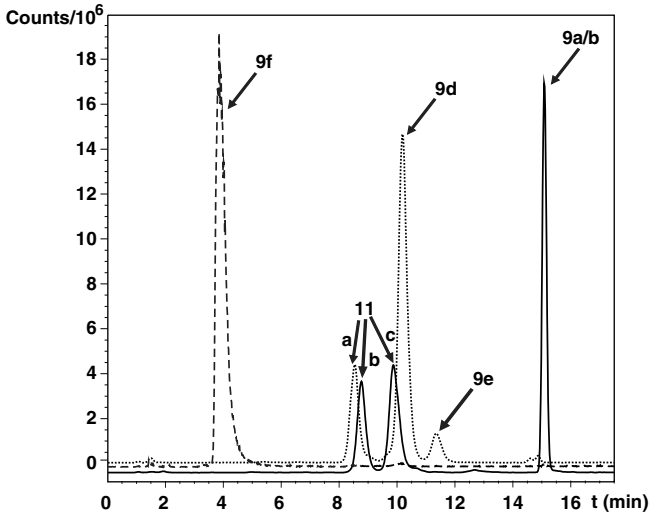

Fig. 3. LC-MS extracted ion chromatograms of a *Ribes uva-crispa* leaf extract. Dashed line corresponds to $m/z = 298$, dotted line to $m/z = 282$ and solid black line to m/z 284. All the peaks shown displayed the characteristic fragment *ion* $m/z = 185$ in MS/MS corresponding to a sodium adduct of glucose; all peaks of $m/z = 282$ and m/z 298 had UV-absorption peaking around 210 nm whereas the peaks of $m/z = 284$ did not have UV-absorption above 200 nm; the peak at $t_R = 15.0$ min was the only to produce the fragment ion $[M–HCN + Na]^+$ in MS/MS.

firmed to be **9f** (Nishida et al., 1994). The other three compounds were hydroxynitrile glucosides, which had not been identified previously (Table 1, Fig. 4). Compound **11a** was the *Z*-isomer of **9d**; the structure elucidation was based on ^{1}H NMR analysis, including NOESY spectrum that showed NOE between the methyl and the methylene group.[1] Compounds **11b** and **11c** were stereoisomeric glucosides of 3-hydroxy-3-methylbutanenitrile, as shown by the presence of two methyl group doublets and two methine resonances appearing as double quartets in their ^{1}H NMR spectra, in addition to signals of β-D-glucopyranose moiety. The parent compound can exist as four stereoisomers (2*R*,3*R*, 2*R*,3*S*, 2*S*,3*R*, and 2*S*,3*S*). However, because the compounds are biosynthesized from L-isoleucine (see radiolabelling experiment below, Section 2.2), which has the 2*S*,3*S* configuration, and formation of **3** from **1** (Fig. 1) does not affect the absolute configuration at C-3 of the latter, they are concluded to be the epimeric 2*R*,3*S* and 2*R*,3*R* forms. Because only very small amounts of material were available, hydrolysis of the glucosides, isolation of their hydroxynitriles, and independent determination of their absolute configuration could not be performed.

[1] Rhodiocyanoside D (**9d**) purified from *Rhodiola sacra* was assigned to be the *E*-isomer of 2-(β-D-glucopyranosyloxy)but-2-enenitrile on the basis of NOESY data (Yoshikawa et al., 1997), but the compound was erroneously given the incorrect configurational descriptor (2*Z*), as was the corresponding aglycone isolated from *Ribes nigrum* (Nishimura et al., 1987). Thus, (2*Z*)-2-(β-D-glucopyranosyloxy)but-2-enenitrile (**11a**) is a new compound.

Table 1
^{1}H NMR data for compounds isolated from *Ribes uva-crispa* leaves[a]

Proton	11a	11b	11c
H-2		2.97 (*qd*, 7.1, 4.4)	3.18 (*qd*, 7.1, 5.4)
H-3	6.75 (*q*, 7.2)	3.96 (*qd*, 6.3, 4.4)	3.92 (*qd*, 6.3, 5.4)
H-4	1.94 (*d*, 7.2)	1.38 (*d*, 6.3)	1.32 (*d*, 6.3)
CH_2 or CH_3	4.40 (*d*, 11.9) and 4.46 (11.9)	1.37 (*d*, 7.1)	1.28 (*d*, 7.1)
H-1′	4.31 (*d*, 7.8)	4.38 (7.7)	4.37 (7.7)
H-2′	3.21 (*dd*, 9.0, 7.8)	3.21 (*dd*, 9.0, 7.8)	3.20 (*dd*, 9.0, 7.8)
H-6′	3.68 (*dd*, 12.2, 5.1), 3.88 (12.2, 2.0)	3.67 (*dd*, 12.2, 5.5), 3.88 (12.2, 2.0)	3.65 (*dd*, 12.2, 4.7), 3.88 (12.2, 2.0)
Remaining glucose protons	3.26–3.45	3.27–3.39	3.26–3.37

[a] In methanol-d_4; δ values relative to residual solvent signal set to $\delta = 3.31$; multiplicity and coupling constants in Hz are given in parentheses.

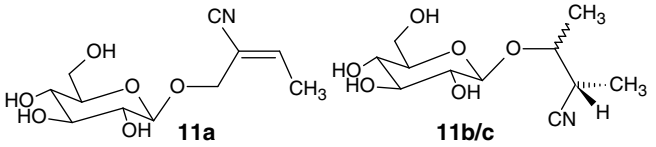

Fig. 4. New compounds isolated from *Ribes uva-crispa* leaves. **11a** = (2*Z*)-2-(β-D-glucopyranosyloxy)but-2-enenitrile, **11b** = (2*R*)-4-(β-D-glucopyranosyloxy)-2-butanenitrile with $t_R = 8.8$ min, **11c** = (2*R*)-4-(β-D-glucopyranosyloxy)-2-butanenitrile with $t_R = 9.9$ min.

Compound **9f** has previously been found in *Ribes fasciculatum* var. *chinense* Max. (Dat et al., 2005) and it has been reported to co-occur with **9e** in *Rhodiola* species (Fan et al., 1999; Ohsugi et al., 1999). Compounds **11a**–**c** have not been reported previously but the corresponding hydroxynitriles were found in *Ribes nigrum* L. (black currant) flower buds along with the hydroxynitrile corresponding to **9d** (Nishimura et al., 1987). In the latter work, the flower buds were extracted with dichloromethane and the hydroxynitriles subsequently purified by distillation (Nishimura et al., 1987). If present, polar and non-volatile glucosides would not have been identified by this method; however, the hydroxynitriles may have arisen by hydrolysis during the initial storing, transportation and processing of the plant material. In accordance with existing names and naming practice of cyanogenic glucosides and related compounds (Lechtenberg and Nahrstedt, 1999) we suggest the following names: rhodiocyanoside E for **11a**, and the names ribesuvanin A and B for the first eluted (**11b**) and the later eluted (**11c**) (2*R*)-2-methyl-3-(β-D-glucopyranosyloxy)butanenitrile, respectively. Determination of their configuration at C-3 has yet to be performed.

2.2. α-, β- and γ-Hydroxynitrile glucosides have common amino acid precursors

In order to elucidate the biosynthetic precursors of the hydroxynitrile glucosides, radiolabelled L-amino acids were

administered to detached leaves. The *R. uva-crispa* and *L. japonicus* leaves metabolized L-[U–^{14}C]isoleucine into **9a/b** as demonstrated by TLC and subsequent LC-MS analysis of radiolabelled compounds (Fig. 5). Furthermore, TLC demonstrated that *R. uva-crispa* leaves synthesized one additional radiolabelled compound from L-[U–^{14}C]isoleucine compared to *L. japonicus* leaves. LC-MS analysis showed that the additional compound was **9f**, consistent with the metabolite profiling, which showed that *L. japonicus* did not contain **9f**. The compounds **9d** and **9e** co-eluted with **11a–c** in the TLC lane with the *R. uva-crispa* extract as demonstrated by LC-MS. The compounds **9d**, **9e** and **11b** were found at the corresponding R_f value in the lane with *L. japonicus* extract. The amounts of **11a–c** found in *L. japonicus* are extremely low (data not shown), which most likely explains why we did not detect **11a** and **11c** in any radiolabelled spots from this extract. It was not possible to separate **9d**, **9e** and **11a–c** from the *R. uva-crispa* extract further by TLC to elucidate whether the radiolabel was incorporated into the new compounds or only **9d** and **9e**. However, administration of L-[U–^{14}C]valine or -leucine to the *R. uva-crispa* and *L. japonicus* leaves did not give rise to radiolabelled compounds at the R_f value of **11a–c**. Thus, isoleucine remains the most likely precursor of **11a–c**.

The *R. uva-crispa* leaves did not synthesize any of the compounds **8** or **10b–g**, whereas L-[U–^{14}C]valine or -leucine was readily converted to respectively linamarin by *L. japonicus* and the assumed leucine-derived compounds by *H. vulgare*. To our knowledge, this is the first demonstration that the non-cyanogenic **10d–g** are in fact leucine-derived like the cyanogenic glucoside epiheterodendrin (**10b**) with which they co-occur in *H. vulgare* (Nielsen et al., 2002). This result emphasizes the validity of the assumption that the compounds **9a–f** and **11a–c** also have a common amino acid precursor. It should, however, be noted that *R. uva-crispa* apparently synthesized a compound derived from L-[U–^{14}C]leucine that co-eluted in TLC with **9a**. Species of *Rhodiola* have been reported to contain the leucine-derived **10a** alongside with the isoleucine-derived compounds (Fan et al., 1999; Nakamura et al., 2007; Ohsugi et al., 1999; Yoshikawa et al., 1997). We could not detect this compound in *R. uva-crispa*, in LC-MS screening analyses or in analyses of the radiolabelled compounds isolated from the TLC-plate. However, *R. uva-crispa* leaves were administered 2.5 times as much L-[U–^{14}C]amino acid as *L. japonicus*, giving rise to increased background signals that may explain the radiolabelled spot originating from L-[U–^{14}C]leucine.

2.3. Presence or absence of β- and γ-hydroxynitrile glucosides is determined by a single recessive genetic trait in L. japonicus

To further establish the connection between the isoleucine-derived compounds we made use of naturally occurring genetic variation in publicly available wild accessions of *L. japonicus* (Legume Base, Miyazaki University, Japan). Metabolite profiling of a large number of accessions disclosed that the MG-74 accession, originally collected from the Japanese town of Uwa in the Ehime prefecture, produces **8** and **9a** but lacks **9d** and **9e**. At the same time, **11a–c** are also absent from this line (Table 2). In order to establish a possible genetic relationship between the co-occurrence of **9d** and **9e** and **11a–c** we crossed the MG-74 accession to one of the most commonly used experimental accessions Miyakojima MG-20. The MG-20 accession contains **8**, **9a**, **9d**, **9e** and **11a–c** (Table 2). The F1 progeny of this cross resembled the MG-20 parental line in that it contained **8**, **9a**, **9d**, **9e** and **11a–c** (data not shown). LC-MS analyses of 108 F2 lines for the presence of **9d**, **9e** and **11a–c** showed a complete correlation between the presence or absence of **9d** and **9e** and the presence or absence of **11a–c** with approximately a quarter of the lines (30 lines) lacking these compounds (Table 2). This data

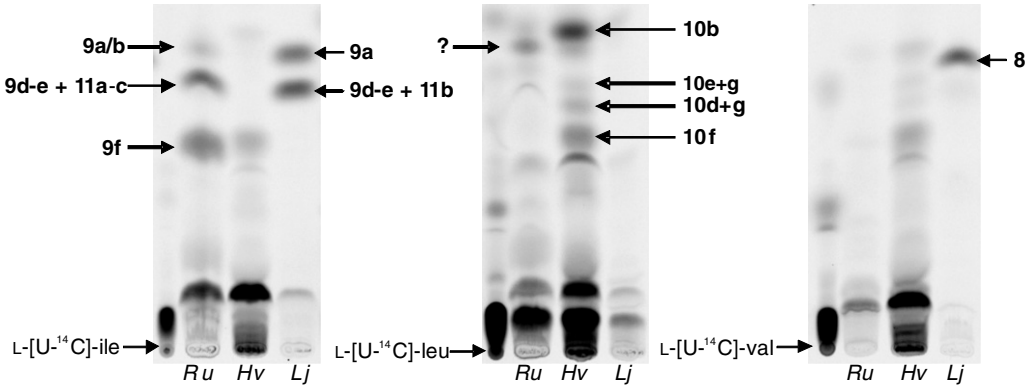

Fig. 5. Amino acid precursors of non-cyanogenic hydroxynitrile glucosides. Radiolabelled products formed upon administration of specific L-[U–^{14}C]amino acids to detached leaves as monitored by radio TLC as described in Forslund et al. (2004). *Ru* = *Ribes uva-crispa*, *Hv* = *Hordeum vulgare*, *Lj* = *Lotus japonicus*. The compounds present in the radiolabelled spots were extracted and identified by LC-MS. The detached leaves of *Hv* and *Ru* were not as biosynthetically active with respect to hydroxynitrile glucosides production as those of *Lj*. To compensate, higher amounts of L-[U–^{14}C]amino acids were administered to leaves of these plants which is reflected by the higher background signal observed in the lanes with *Hv* and *Ru* extracts. The compound marked "?" could not be differentiated from **9a/b**.

Table 2
Relative distribution of valine- and isoleucine-derived hydroxynitrile glucosides in different genera and species

Plant (number of plants/ cultivars/ species)	Relative amounts (average % of total amount of hydroxynitrile glucosides (SD[a]))				
	Compounds				
	8	**9a/b**	**9d + 9e**	**9f**	**11a–c**
Lotus leaves					
conimbricensis (2)	0.4 (0.5)	16.2 (1.1)	82.6 (1.2)	0	1.0 (0.1)
japonicus MG-20 (2)	1.2 (0.4)	41.1 (6.1)	55.2 (6.1)	0	2.2 (0.4)
japonicus MG-74 (3)	0.9 (0.2)	99.1 (0.2)	0	0	0
MG-74 × MG-20 *F2* (78)	0.9 (0.5)	56.0 (11.3)	41.3 (10.9)	0	1.6 (0.5)
MG-74 × MG-20 *F2* (30)	0.9 (0.5)	99.1 (0.6)	0	0	0
Ribes leaves					
uva-crispa[b] (7)	0	11.9 (4.9)	12.4 (4.1)	61.9 (12.5)	13.8 (7.9)
Other species[c] (4)	0	15.9 (1.6)	24.6 (5.2)	45.4 (6.8)	14.0 (3.4)
Rhodiola root					
rosea (8)	0	8.1 (3.0)	74.0 (9.1)	2.9 (3.3)	14.6 (6.7)
dumulosa (1)	0	90.1	9.9	0	0
heterodonta (1)	0	76.8	23.2	0	0
wallichiana (1)	0	22.6	0	0.0	77.4
kirilowii (S1946-1567) (1)	0	43.6	2.8	0	53.6
kirilowii (P1976-5247) (1)	0	0	23.4	4.6	72.0
semenovii (1)	0	14.1	77.2	0.9	7.8
yunnanensis (1)	0	11.1	72.1	4.9	12.0

[a] SD = standard deviation of the average concentrations in different plants/cultivars/species.
[b] Plants sampled from the Copenhagen University Pometum.
[c] *R. nigrum* (1), *R. rubrum* (1), *R. nidigrolaria* (1), *R. uva-crispa* (1) sampled from a garden in the greater Copenhagen area.

suggest that a single recessive genetic trait is responsible for the lack of **9d** and **9e** as well as **11a–c** in the MG-74 accession. This genetic co-segregation supports the notion of a tight connection between the biosynthesis of the isoleucine-derived **9d** and **9e** and the compounds **11a–c**.

Nielsen et al. (2002) suggested that the biosynthesis of the β- and γ-hydroxynitrile glucosides in *H. vulgare* is carried out by the cyanogenic glucoside pathway (Fig. 1). It was proposed that the putative multifunctional CYP71 ortholog is a promiscuous enzyme able to hydroxylate any carbon of the putative leucine-derived **3** followed by dehydrations and/or multiple hydroxylations leading to the **10b–g** hydroxynitriles. The known leucine-derived hydroxynitrile glucosides (Lechtenberg and Nahrstedt, 1999) represent all possible hydroxylations of the leucine-derived **3**. With the identification of **11b** and **11c** the series of isoleucine-derived compounds now also represents all possible hydroxylations of the isoleucine-derived **3**. Knowing that isoleucine-derived **2** is likely to be a common intermediate in biosynthesis of **9a**, **9d** and **9e** (Morant et al., 2007) it is reasonable to assume that all isoleucine-derived hydroxynitriles are indeed produced by cytochrome P450 enzymes derived from the CYP71 catalyzing the conversion from oxime to α-hydroxynitrile. Thus, a possible explanation for the demonstrated inheritance of the ability to produce β- and γ-hydroxynitrile glucosides in *L. japonicus* is that the MG-20 accession has two active CYP71 paralogs: one putative CYP71 strictly catalyzing the production of α-hydroxynitriles ($CYP71_{\alpha}$) leading to **8** and **9a** and an additional CYP71 producing β- and γ-hydroxynitriles ($CYP71_{\beta\gamma}$), the latter being absent or inactive in the MG-74 accession. In support of the presence of $CYP71_{\alpha}$ and $CYP71_{\beta\gamma}$ paralogs, we discovered that one of two analyzed accessions of *Rhodiola kirilowii* (Regel) Maxim contained the non-cyanogenic compounds while being devoid of **9a/b** (Table 2), thus apparently only possessing $CYP71_{\beta\gamma}$ activity. Another possible explanation that would satisfy the inheritance observed in *L. japonicus*, could be the presence of a transcription factor that co-regulates several enzymes responsible for β- and γ-hydroxynitrile biosynthesis.

2.4. Occurrence and ratios of α-, β- and γ-hydroxynitrile glucosides in different genera

The qualitative and quantitative analyses of the hydroxynitrile glucoside distribution within different species of *Ribes*, *Rhodiola* and *Lotus* further support the proposed connection between the biosynthetic pathways of cyanogenic and non-cyanogenic hydroxynitrile glucosides. We found isoleucine-derived β- and γ-hydroxynitrile glucosides co-occurring with α-hydroxynitrile glucosides in all investigated *Ribes* and *Rhodiola* species and in two species of *Lotus* (Table 2). The only component in LC-MS analyses of *Rhodiola* extracts corresponding to a cyanogenic compound was found at the t_R of **9a/b**. An authentic standard of **10a** was not available but the t_R of **10b** from *H. vulgare* was 2 min longer than that of **9a/b** (data not shown). We therefore consider it highly unlikely that **10a** and **9a/b** co-elute in the LC-MS analysis and accordingly assume that the cyanogenic constituent in *Rhodiola* species is **9a/b**. Our results for *Jatropha* species were inconclusive. Van den Berg et al. (1995) reported the finding of **9e** in latex of *Jatropha multifida* L. We saw peaks corresponding to the presence of **9a/b, 9d** and **11a-c** in LC-MS analyses of leaf extracts of *J. podagrica* Hook but all compounds were not found in all replicates and none were detected in two other available species.

Within species and cultivars of the genus *Ribes* and within accessions of *Rhodiola rosea* and *L. japonicus* MG-74 × MG-20 *F2*, the ratios between α-, β- and γ-hydroxynitrile glucosides showed very little variation. Furthermore, these ratios showed the same trend, with the genus *Ribes* and some species of the two other genera being completely dominated by γ-hydroxynitrile glucosides (Fig. 6). This suggests a biological significance of the non-cyanogenic

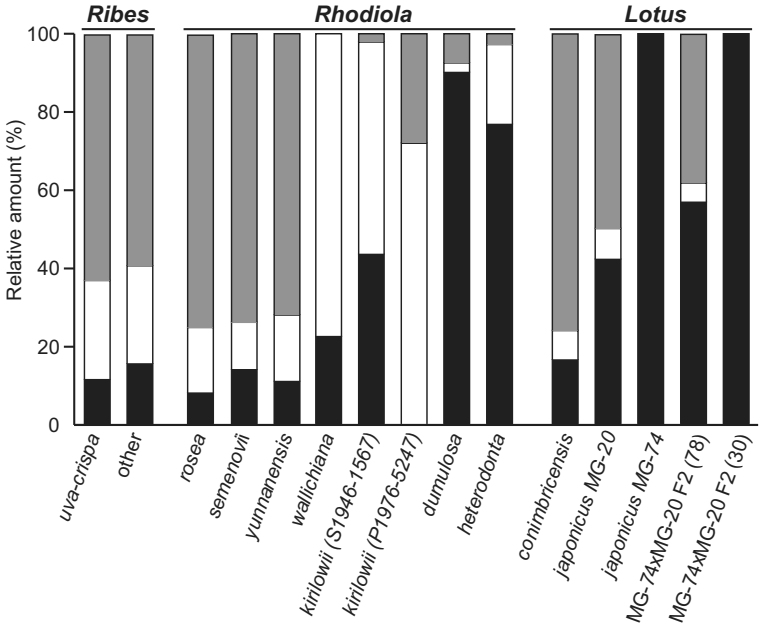

Fig. 6. Relative distribution of α-, β- and γ-hydroxynitrile glucosides in different species and genera. The relative distribution of compounds from Table 2 as they group into α-, β- and γ-hydroxynitrile glucosides; black = α, grey = β and white = γ.

compounds and a tight regulation of the ratios between the different structures. On the other hand, some *Rhodiola* species were devoid of γ-hydroxynitrile glucosides and the distribution between α- and β-hydroxynitrile glucosides appeared variable. This variability may be explained by further metabolism of **9e** and **9f** as several derivatives have been identified in different plants: a **9f** 2,3-epoxide was isolated from *Sedum cepaea* L. aerial tissue (Crassulaceae) (Nahrstedt et al., 1982), and in total five different **9f** aryl esters have been isolated from roots of *Rhodiola quadrifida* (Pall.) Fisch. et Mey. (Yoshikawa et al., 1996), *Ribes nigrum* seed (Lu et al., 2002) and pulp of processed fruits from *Ribes rubrum* L. (red currant) (Schwarz and Hofmann, 2007). We did not find these derivatives in any of the plants studied but it is possible that these more apolar compounds have escaped detection by the LC-MS method employed.

Only in *Lotus* species did the valine-derived **8** co-occur with isoleucine-derived β- and γ-hydroxynitrile glucosides, however in extremely low amounts (Table 2). In other plant species where **8** and **9a** co-occur while no β- and γ-hydroxynitrile glucosides can be detected, e.g. *Trifolium repens* L. (white clover), *Linum usitatissimum* L. (flax), *Manihot esculenta* Crantz (cassava) and *Lotus corniculatus*, *Lotus arabicus* and *Louts tenuis* L., **8** constitutes 20–93% of the total amount of cyanogenic glucosides (Collinge and Hughes, 1982; Lykkesfeldt and Møller, 1995; Niedzwiedz-Siegien, 1998; Zagrobelny et al., 2007a,b). This apparent inverse relationship between the occurrence of the valine-derived cyanogenic glucoside **8** and the isoleucine-derived β- and γ-hydroxynitrile glucosides further accentuates the notion that the latter are important compounds for the plants. Why **8** is apparently becoming obsolete or counter selected during the evolutionary process leading to the ability to produce β- and γ-hydroxynitrile glucosides is currently not understood.

3. Conclusions

We have identified previously unknown compounds in the group of β- and γ-hydroxynitrile glucosides and demonstrated that the non-cyanogenic compounds of *R. uva-crispa*, *H. vulgare* and *L. japonicus* are derived from the same amino acids as the co-occurring cyanogenic glucosides. Furthermore, our findings provide further support that these non-cyanogenic compounds are produced by diversification of the biosynthetic pathway for cyanogenic glucosides, possibly mediated by the ability of the putative CYP71-paralogs to perform multiple hydroxylations and dehydrations. The β- and γ-hydroxynitrile glucosides are strongly dominant and the ratios between structures apparently tightly regulated in the plants where they occur. This indicates that the β- and γ-hydroxynitrile glucosides are biologically important although their function is currently unclear.

4. Experimental

4.1. General experimental procedures

4.1.1. Extraction of plants for LC-MS analysis

For profiling of hydroxynitrile glucoside content either whole leaves (*Ribes*: fully unfolded young leaves; *Lotus*: first unfolded leaf from the shoot apex), or whole small

roots or pieces of large roots homogenized in liquid nitrogen were boiled for 3 min in 80% (v/v) MeOH and cooled on ice. The extracts were either lyophilized, resuspended in water and filtered, or only filtered (0.45 μm PVDF spin filter (*Millipore*), 10 min, 10,000 g). The concentrations of hydroxynitrile glucosides were determined by LC-MS analysis.

4.1.2. Crude isolation of hydroxynitrile glucosides

Compounds **9a**, **9d** and **9e** were isolated from *L. japonicus* leaves and flowers (150 g). Plant material was extracted as described above and the extract concentrated on a rotary evaporator. The concentrate was redissolved in water, extracted with three volumes of *n*-pentane, boiled with activated charcoal, cooled and filtered through a layer of sand and silica gel (*Silica 60* particle size 0.040–0.063, 230–400 mesh ASTM, *Merck*). The filtrate was dried together with silica gel on rotary evaporator (0.05 g/g plant material extracted). The silica gel coated with evaporation residue was packed on top of a silica gel column (0.3 g/g plant material extracted). The column was first eluted with CH_2Cl_2 (7 ml/g silica) which was discarded. Then the column was eluted with 9:1 CH_2Cl_2:MeOH. Compounds **9a**, **9d** and **9e** were eluted together in the 700–1200 ml fraction. **9a/b**, **9f** and **11a-c** were isolated in a similar manner from 45 g leaves of *R. uva-crispa* cv. "Invicta". Compounds **11a-c** were eluted together with **9a/b**, **9d** and **9e**, followed by **9f**. In both cases, the relevant fractions were combined and evaporated to an almost colorless syrup. This syrup was redissolved in water and further fractionated using preparative LC-MS.

4.1.3. LC-MS analyses and purification by preparative LC-MS

Screening analyses: Separation was carried out on a Synergy Fusion column from *Phenomenex* (150 × 2 mm, 4 μm particles, 0.3 ml/min) fitted on a *Dionex* HPLC with HPLC pump (*Dionex* P680), autosampler (*Dionex ASI-100*), UV-detector (*Dionex UVD340U*) and column oven set to 25 °C (*Dionex STH585*). The mobile phases were A: 0.1% (v/v) HCO_2H, 50 μM NaCl, 2% CH_3CN and B: 0.1% (v/v) HCO_2H, 50 μM NaCl, 50% CH_3CN. The gradient program was as follows: 0–13 min, 0% B; 13–30 min, linear gradient 0–100% B followed by wash and equilibration. The flow was passed directly from the UV-detector to the MS-detector, a Thermo Finnigan MSQ single quadropol operated in electrospray mode with positive ionization (ionization temperature 365 °C, cone voltage 2.5 kV). Hydroxynitrile glucosides are not easily ionized themselves but in general glucosides readily form adducts with Na^+ and other ions present during electrospray formation. To favor Na^+ adduct formation, NaCl was added to the mobile phases. Ionization efficiency proved to be similar for **8** (*A.G. Scientific*), **9a** and **9e** (purified from *L. japonicus*) and a mixture of **9a** and **9b** (*Toronto Research Chemicals*) (data not shown). Therefore, all hydroxynitrile glucosides were quantified by a standard curve prepared from **9a** (linear from 2 to 250 μM). For increased reproducibility and accuracy, the cyanogenic glucoside amygdalin (*Sigma*), was added as an internal standard to samples and standards at a level of 100 μM. Diagnostic LC-MS analyses with ion trap MS detection were carried out using an *Agilent* 1100 Series LC linked to a *Bruker* HCT-*Plus* ion trap mass spectrometer. Separation was carried out using a *Synergy Fusion*-RP column from *Phenomenex* (100 × 2 mm, 2 μm particles, flow rate 0.2 m/min). The mobile phases were A: 0.1% (v/v) HCO_2H, 50 μM NaCl and B: CH_3CN with 0.1% (v/v) HCO_2H. The gradient program was as follows: 0–7.5 min, linear gradient 3–24% B; 7.5–10 min, linear gradient 24–100% B, followed by wash and equilibration. The mass spectrometer was operated in positive ion mode and hydroxynitrile glucosides were detected as Na^+-adducts. Isolation of compounds was performed on the same LC-MS system as the screening analyses. Separation was carried out on a *Synergy Fusion*-RP column from *Phenomenex* (150 × 10 mm, 4 μm particles, 6 ml/min). The mobile phases were A: 0.1% (v/v) HCO_2H, 2% (v/v) CH_3CN and B: 0.1% (v/v) HCO_2H, 50% (v/v) CH_3CN and the gradient program as follows: 0–13 min, 0% B; 13–30 min, concave gradient 0–100% B, followed by wash and equilibration. A constant flow split was used to direct approximately 0.2 ml/min of the flow to the MS-detector where the hydroxynitrile glucosides were detected as Na^+-adducts after addition of 250 μM NaCl at 50 μl/min. The remainder of the flow was directed to the UV-detector and subsequently to an automatic fraction collector (*Foxy Jr*).

4.1.4. Metabolism of L-[U-^{14}C] amino acids

Administration (24 h) of either L-[U-^{14}C]valine, -isoleucine, -leucine or, for reference, H_2O (from *Millipore*, UV-lit and filtered, 0.22 μm) to freshly detached leaves of *L. japonicus*, *R. uva-crispa* and *H. vulgare*, extraction and subsequent TLC-analysis of the radiolabelled biosynthetic products was performed as described in Forslund et al. (2004). *L. japonicus* apical leaves were administered 18.5 Bq radionuclide activity per leaf. *R. uva-crispa* young unfolded leaves and *H. vulgare* seedlings were supplied 46.25 Bq per leaf. All extracts were lyophilized and redissolved in 25 μl H_2O. In the case of *H. vulgare* three replicate extracts were combined before lyophilization. For TLC-analysis, 10 μl of sample solutions and, for reference, 3.7 Bq L-[U-^{14}C]amino acids was used.

4.1.5. Structure determination by NMR

The structures of **9a**, **9d** and **9e** purified from *L. japonicus* were confirmed by ^{1}H and ^{13}C NMR spectra recorded on a *Bruker Advance* 400 MHz spectrometer in methanol-d_4 or D_2O, using a 5 mm probe. Structures of **11a-c** purified from *R. uva-crispa* were determined by ^{1}H NMR, COSY and NOESY (600 ms mixing time) spectra recorded with a *Bruker Avance* 400 MHz spectrometer equipped with 1 mm probe, using solutions in methanol-d_4. The same equipment was used for confirmation of the structure of **9f.**

4.1.6. L. japonicus segregation study

The MG-74 accession was crossed to Miyakojima MG-20. The hybrid nature of the F1 progeny lines ($n = 6$) was confirmed using the TM0707 genetic SSLP marker (http://www.kazusa.or.jp/lotus), which showed a length polymorphism between the MG-20 and MG-74 accessions. The F1 progeny lines were allowed to self giving rise to the F2 progeny lines.

4.2. Plant material

4.2.1. Self-grown plants

L. japonicus cv. MG-74 and MG-20 were germinated from seed on filter paper and grown in soil in a greenhouse at 22 °C. *H. vulgare* cv. "Mentor" was germinated from seed in soil and grown in a greenhouse at 15–18 °C.

4.2.2. Plants sampled in Copenhagen University Botanical Garden

Lotus conimbricensis Brot. (accession no. S1927-0652), *Rhodiola rosea* L. (accessions P1991-6442, P0000-5077M and F, P2003-5027, P1991-5336M and F, P1997-5369, P1988-5423 and P1977-5851), *R. dumulosa* (Franch.) S.H. Fu (P0000-5076), *R. wallichiana* (Hook.) S.H. Fu (P0000-5078), *R. heterodonta* (Hook. fil. & Thomson) Boriss. (P1970-5211), *R. kirilowii* (Regel) Maxim. (S1946-1567 and P1976-5247), *R. semenovii* (Regel & Herder) Boriss. (S1962-2223) and *R. yunnanensis* (Franch.) S.H.Fu (P0000-5079) and *Jatropha integerrima* Jacq. (P1980-5820), *J. platanifolia* Standl. (S1993-0253) and *J. podagrica* Hook (S1966-2217).

4.2.3. Plants sampled in the Copenhagen University Pometum

R. uva-crispa cv. "American Mountain", "Captivator", "Flamingo", "Larell", "Miezuriniac", "Red Hinomaki" and "Whitesmith".

4.2.4. Plants sampled in a private garden in the greater Copenhagen area

R. uva-crispa cv. "Invicta", R. nigrum cv. "Ben Alder", R. rubrum cv. "Stanza" and R. nidigrolaria (nigrum × uva-crispa) cv. "Jostabeer".

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