Volatile metabolite emission

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Biochemical Processes and Macromolecular Structures
A role for differential glycoconjugation in the emission of phenylpropanoid volatiles from tomato fruit discovered using a metabolic data fusion approach

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Abstract

A role for differential glycoconjugation in the emission of phenylpropanoid volatiles from ripening tomato fruit (Solanum lycopersicum L.) upon fruit tissue disruption has been discovered in this study. Application of a multi-instrumental analytical platform for metabolic profiling of fruits from a diverse collection of tomato cultivars revealed that emission of three discriminatory phenylpropanoid volatiles, namely methyl salicylate, guaiacol and eugenol, took place upon disruption of fruit tissue through cleavage of the corresponding glycoconjugates, identified putatively as hexose-pentosides. However, in certain genotypes, phenylpropanoid volatile emission was arrested due to the corresponding hexose-pentoside precursors having been converted into glycoconjugate species of a higher complexity: dihexose-pentosides and malonyldihexose-pentosides. This glycoside conversion was established to occur in tomato fruit during the later phases of fruit ripening and has consequently led to the inability of red fruits of these genotypes to emit key phenylpropanoid volatiles upon fruit tissue disruption. This principle of volatile emission regulation can pave the way to new strategies for controlling tomato fruit flavor and taste.

Key words: metabolomics, tomato, phenylpropanoid, volatile, data fusion, glycoside
Introduction

More than 7,000 metabolites, including volatiles have already been identified in plant-based foods and beverages (Goff and Klee, 2006). Volatile organic compounds (VOCs) constitute a significant part of the plant metabolome and the number of individual volatiles already described for various plants is approaching 2,000 (Dudareva et al., 2006). Significant progress has been made on the functional characterization of these plant volatiles over the past decades. For example, volatiles have been shown to play an important role in the interaction between plants and their environment. They are involved in the defense of plants against pathogens where they serve as airborne signaling molecules to induce a defense response in other plant parts or neighboring plants (Shulaev et al., 1997). They also act as direct repellents of herbivorous pests or as attractants of the predators of these pests as part of the “cry for help” response (Dudareva et al., 2004; Kappers et al., 2005; Baldwin et al., 2006). In addition, flower volatiles are important for the attraction of pollinators (Dudareva et al., 2004) while fruit volatiles may have a role in attracting seed dispersers (Goff and Klee, 2006; Schwab et al., 2008). Besides their physiological and ecological functions, plant volatiles are also important determinants of consumer quality traits in flowers, fruits and vegetables as well as the processed products derived therefrom.

Tomato is one of the most important vegetable crops worldwide and its fresh fruits and processed products are consumed and appreciated in every society. Volatiles are considered as major determinants of tomato fruit flavor (Buttery et al., 1987; Buttery and Ling, 1993; Baldwin et al., 1998; Baldwin et al., 2000; Tandon et al., 2000; Krumbein et al., 2004; Ruiz et al., 2005; Tieman et al., 2006; Zanor et al., 2009; Kovács et al., 2009). Several hundred tomato fruit volatile compounds have been described in the literature (Petro-Turza, 1987), but only a small part of this diversity is believed to have an impact on tomato fruit organoleptic properties (Buttery and Ling, 1993; Baldwin et al., 2000). We have previously screened red-ripe fruits for variation in their volatile metabolome using a collection of 94 tomato cultivars representing the current diversity within commercial germplasm (Tikunov et al., 2005). In that study, three phenylpropanoid (PhP) volatiles: methyl salicylate (MeSA), guaiacol and eugenol, were found to be discriminatory within this germplasm collection and roughly divided the cultivars into two groups. Fruits from one group had the capacity to emit significant amounts of these three PhP volatiles upon fruit tissue disruption (blending), while fruits from the other group emitted none or hardly any.

The considered relevance of these findings relates to their potential importance in consumer perception of fruit taste differences. It has been proposed previously that PhP volatiles likely have an impact on tomato fruit aroma. Methyl salicylate, the methyl ester of salicylic acid, is a potent odor component of wintergreen. Methyl salicylate content has been shown to be negatively correlated with typical tomato flavor (Krumbein and Auerswald, 1998). Guaiacol is also a well known flavoring
compound and has been associated with a so-called “pharmaceutical” aroma in tomato fruits (Causse et al., 2002). Likewise, eugenol is a well known odorant which gives the distinctive, pungent flavor to cloves and significantly contributes to the aroma of cinnamon. Although a potential physiological role for these PhP volatiles in tomato fruits remains unclear, they have been implicated to have a signaling and/or defense function (Shulaev et al., 1997; Koeduka et al., 2006; Sasso et al., 2007). There is therefore clear potential for the release of these compounds to influence either negatively or positively, tomato flavour.

In plants, phenylpropanoid volatiles are primarily derived from phenylalanine (Dudareva and Pichersky, 2000). Cinnamic acid, directly derived from phenylalanine by a de-amination catalyzed by phenylalanine ammonia lyase (PAL), can either be \( \beta \)-oxidatively or non-oxidatively converted into benzoic acid. This can be further hydroxylated into salicylic acid (SA) by benzoic acid 2-hydroxylase. Recently, genetic studies in *Arabidopsis* revealed the existence of an alternative pathway for the production of salicylic acid from isochorismate, thus by-passing phenylalanine and its derivatives (Wildermuth et al., 2001). Salicylic acid is the immediate precursor of the volatile methysalicylate, through the action of salicylic acid methyl transferase (SAMT) (Boatright et al., 2004). Cinnamic acid can also be converted to other phenolic acids: \( p \)-coumaric acid, caffeic and ferulic acid. Ferulic acid can be converted into coniferyl alcohol and further to eugenol (Gang, 2005). The biochemical origin of guaiacol in plants is not completely known. However, its chemical structure clearly points to the same phenylpropanoid origin, as has already been demonstrated for bacteria (Chang and Kang, 2004).

Glycosylation is a common means to conjugate plant secondary metabolites, in order to facilitate their transport and storage and to reduce their reactivity by blocking reactive hydroxyl groups. In tomato fruit, many volatile compounds, including PhP volatiles, are bound as glycosides, thus representing an aroma reserve in tomato fruit (Buttery et al., 1990; Marlatt et al., 1992; Ortiz-Serrano and Gil, 2007). Such glycosidically-bound volatiles can be liberated when cell compartmentation is destroyed, as happens on e.g. consumption of fresh fruits or industrial processing or as may happen during late ripening stages. As a consequence of this disruption, the contents of different cell compartments can mix and stored volatile glycosides become exposed to endogenous or exogenous cleavage enzymes, such as glycosyl hydrolases (glycosidases), which leads to glycoside cleavage and volatile emission. Thus, understanding the biochemical processes leading to the formation and/or cleavage of volatile glycoconjugates may provide tools to exploit more efficiently the aroma reserve present in tomato fruit in order to improve tomato fruit flavor.

In order to gain greater insight into the volatile compound variation in tomato fruit, we previously analyzed the volatile metabolites using SPME-GC-MS, in a broad screening of 94 contrasting tomato genotypes representing the variation present in the germplasm of commercial tomato varieties (Tikunov et al., 2005; Van Berloo et al., 2008). This non-targeted metabolomics approach enabled the detection and putative identification of 322 volatiles. Subsequent
multivariate analysis revealed: (i) differences between tomato types (cherry vs. round tomatoes) driven by the accumulation of phenolic-derived volatiles such as phenylethanol and phenylacetaldehyde; (ii) that the phenylpropanoid-derived volatiles methylsalicylate, guaiacol and eugenol roughly split the set of genotypes into two distinct groups, independent of tomato fruit (pheno)type, where fruits of one of these groups emitted considerable amounts of these three PhP volatiles, whereas fruits of the other emitted little or none.

In the present paper we describe the investigation into the biochemical basis underlying this difference in capacity to emit PhP volatiles. A multi-instrumental metabolomics platform was used to profile fruits of the same broad tomato germplasm collection for both volatile and non-volatile metabolites. Metabolic data fusion of both LC-MS and GC-MS datasets, followed by multivariate analyses suggested a principal for the regulation of PhP volatile emission in tomato fruit through differential volatile-sugar conjugation patterns. Subsequent series of quantitative biochemical experiments proved an important role of this process in regulating the emission of phenylpropanoid volatiles from tomato fruit.

Results

Approach to elucidate pathways leading to flavor-related volatiles in tomato

Volatile metabolites are generally produced from non-volatile precursors. In order to get more insight into the regulation and dynamics of volatile biosynthesis pathways in tomato fruit, we aimed to compare the metabolite profiles of volatile compounds with those of non-volatile compounds, by analyzing and comparing the fruit metabolic composition of fruit materials of differing origin: a major sample set of 94 cultivars; a number of in vitro enzymatic assays; and a multigenotypic fruit ripening series. Both GC-MS (volatiles) and LC-QTOF-MS (non-volatile, semi-polar compounds) approaches were used. The fruits analyzed were from the same collection of 94 contrasting tomato genotypes as described previously, grown in a replicate trial (Tikunov et al., 2005).

Volatile metabolites were analyzed using headspace SPME-GC-MS. Our sample preparation procedure involved a thawing of initially frozen ground tomato fruits for 10 minutes at 30°C, prior to analysis. This allows the action of endogenous fruit enzymes that are induced, activated or brought into contact with their substrates upon breakdown of cell compartmentation and is meant to resemble processes induced upon fruit blending – a term used further in the text as a simplified reference to the procedure. After sample analysis, the GC-MS chromatograms were subjected to full mass spectral alignment using MetAlign™ software (www.metalign.nl) followed by the filtering out of low intensity ion fragments. Multiple mass signals derived from the
same compound were grouped according to an in-house developed Multivariate Mass Spectral Reconstruction (MMSR) software, as described previously (Tikunov et al., 2005), which resulted in the detection of 217 volatiles each containing more than 5 fragment ions in their mass spectrum. The volatiles detected and putatively identified on the basis of their fragment masses are listed in the Supplemental data. The identities and intergenotypic profiles of 42 volatiles, including methyl salicylate, guaiacol and eugenol, confirmed the results obtained in our previous trial (Tikunov et al., 2005).

Semi-polar, non-volatile metabolites were analyzed by LC-QTOF-MS of methanolic extracts from the same tomato fruit samples. In contrast to the procedure used for the detection of volatiles, the methanolic extracts were directly prepared from frozen fruit powder without any prior incubation or thawing. Thus, since fruits were frozen in liquid nitrogen within a few seconds and stored at -80°C before analysis, sample composition resembles the metabolic composition of intact tomato fruit. Like the GC-MS chromatograms, the LC-MS chromatograms were subjected to full mass spectral alignment using the MetAlign™ software package followed by filtering out low intensity ion fragments. This revealed a data matrix of 1415 ion fragments x 94 samples analyzed. The MMSR performed on this data set resulted in 386 ion fragment clusters, predicted to represent 386 different compounds. The most abundant ion fragment within each cluster was selected as representative of each compound and was used for further analyses. Individuals in this large group of putative compounds were not subjected to prior identification and were thus initially treated as unknowns.

**Fusion of volatile and non-volatile data**

In order to find correlations between volatile and non-volatile metabolites, low-level data fusion was performed by concatenation of both LC-MS and GC-MS normalized data sets. This resulted in a data matrix of 94 genotypes x 603 putative metabolites. A Principal Components Analysis (PCA) was performed on the fused data set (Figure 1). The genotype clustering obtained was similar to that observed previously using GC-MS data only (Tikunov et al., 2005): the first principal component (PC1) revealed differences determined by tomato type (i.e. cherry vs. the rest) and described 17.6% of the variation in the data, while the second principal component (PC2) divided the genotype set into two distinct groups independent of tomato type (Figure 1A). This dichotomy described 16.4% of the total data variation and was determined to be primarily linked to the ability / inability to emit phenylpropanoid-derived volatiles, such as methyl salicylate (MeSA), guaiacol and eugenol (Figure 1B, group GC1). In the fused dataset these three volatile compounds were clearly observed to be associated with a number of LC-MS-detected compounds (Figure 1B, group LC1). In addition, another group of LC-MS derived compounds
appeared as the second determinant of PC2 and showed a negative correlation with the PhP volatiles and their co-correlating non-volatile compounds (Figure 1B, group LC2). According to the Student’s t-test performed, metabolites of all three groups (GC1, LC1 and LC2) were significantly different (p < 0.00001) between the fruit groups differentiated by PC2 (Figure 1A).

The fusion of GC-MS and LC-MS data has identified two distinct genotypic groups: one group was able to emit PhP volatiles from blended fruit tissue and showed a high abundance of the non-volatile compounds belonging to the LC1 group and the complete absence of compounds belonging to the LC2 group. The other group had an abundance of non-volatile compounds of the LC2 group, while PhP volatiles and the LC1 non-volatile compounds were virtually absent. To address a possible relationship between these metabolites, the correlating non-volatile compounds were subjected to further identification.

PhP-volatile emission correlates with the presence of corresponding PhP-V hexose-pentosides

In order to shed light on the identity of the non-volatile compounds correlating with the known PhP volatiles, the LC-MS signals of the putative compounds belonging to the LC1 group were analyzed in more detail. Eugenol, as measured by GC-MS, revealed the strongest correlation (r = 0.8; p < 0.00001) to the non-volatile compound LC1.1 of the LC1 group (eluting at retention time 29.52 min and with a selective nominal ion fragment of m/z 503; Table 1). According to MMSR results, this m/z 503 was found to correlate strongly with several other ions, of which m/z 457 and m/z 293 were the most abundant (Table 1). By applying accurate mass calculations and LC-MS/MS on selected ions, mass 503 (observed accurate m/z = 503.1747) appeared to be the formic acid adduct (FA; mass 46.0055) of the ion with m/z 457 (observed mass 457.1709). Subtraction of correlating fragment m/z 293 (accurate mass 293.0868) from selected ion m/z 457 gave a neutral mass loss of 164.0841. This corresponds closely to the molecular mass of eugenol (-2.4 ppm deviation from calculated mass). Indeed, a fragment ion with an accurate mass of 163.0766, corresponding to within 1 ppm of that of eugenol ([M – H]– = 163.0765) was detected using LC-MS/MS. These results suggest that compound LC1.1 represents a conjugated form of eugenol. The same calculations and identification strategies were performed on the remaining two compounds, LC1.2 and LC1.3 (Table 1), which each also correlated strongly with a PhP volatile. This resulted in two mass spectral models similar to LC1.1: (i) compound LC1.2 eluting at 19.25 minutes represents a methylsalicylate conjugate (LC1.2 correlated with m/z 491 and m/z 293; application of the above fragmentation analysis model gave m/z 491 - m/z 46 (FA) = m/z 445 – m/z 293 = 152 – the molecular mass of methyl salicylate); (ii) compound LC1.3 eluting at 16.22 minutes represents a guaiacol conjugate (m/z 463 – m/z 46 (FA) = m/z 417 – m/z 293 = m/z 124.
– the molecular mass of guaiacol). These results suggest that the three compounds detected by LC-MS, correlating to the three PhP volatiles, represented their respective conjugates, each modified in a similar way with a linkage to a fragment of m/z 293. The observed exact masses, ranging from 293.0868 to 293.0884, have on average a 2.1 ppm deviation from the elemental composition C_{11}H_{18}O_{9}. This fragment ion therefore appears to be a hexose-pentose diglycosidic moiety: [(hexose – H_{2}O) + (pentose – H_{2}O)]^{–}. Indeed, in some MS/MS experiments we could also observe the corresponding hexose and pentose fragments with m/z 162 and 132 (low abundances of these ions did not allow accurate mass calculation), respectively (data not shown). None of the remaining compounds of the LC1 group revealed any relation to guaiacol, methyl salicylate or eugenol according to MS and MS/MS fragmentation patterns.

Low PhP-V tomato fruits contain PhP volatiles bound as complex triglycosides

The group of genotypes whose fruits did not emit PhP volatiles upon blending and did not contain the corresponding hexose-pentosides had significant amounts of non-volatile compounds of the LC2 group from the LC-MS dataset (Figure 1). To shed light on their possible structure, these compounds were also further subjected to LC-QTOF-MS/MS fragmentation analyses. Firstly, it was checked whether fragment ions of the volatile PhP aglycones were present in the MS/MS data. The MS/MS fragmentation of the fragment ion m/z 665 eluting at 17.16 minutes (compound LC2.2, Table 1) revealed a fragment ion of [M – H]^{–} = 123.0445, corresponding to the elemental composition of guaiacol (123.0452), and a fragment ion of m/z 579, corresponding to the loss of a malonyl group ([M – H_{2}O] = 86) and leaving an elemental composition corresponding to guaiacol bound to a dihexose-pentose sugar moiety. Likewise, the observed LC-MS/MS fragments of the compounds LC2.4 eluting at 18.61 minutes and LC2.5 eluting at 27.25 minutes corresponded to methylsalicylate malonyl-dihexose-pentoside and eugenol malonyl-dihexose-pentoside, respectively (Table 1). Compound LC2.1 eluting at 13.58 minutes (parent m/z 579) appeared to be guaiacol dihexose-pentoside. Its MS/MS fragmentation pattern revealed fragments corresponding to dihexose-pentoside (m/z 455), hexose-pentoside (m/z 293) and dihexose (m/z 323), but the loss of the malonyl group was not observed (Table 1). The non-malonylated dihexose-pentosides of methyl salicylate and eugenol were also identified (Table 1, compounds LC2.3 and LC2.6, respectively). Analysis of MS/MS fragmentation of the other compounds of the LC2 group revealed no relation to the PhP volatiles. A potential casual relationship between the presence of malonyl-dihexose-pentosides and the absence of volatile emission was therefore investigated.
For simplicity, the hexose-pentosides of phenylpropanoid volatiles (PhP-V) found in high PhP-V emitters will below subsequently be generally referred to as ‘diglycosides’ and both the dihexose-pentosides and malonyl-dihexose-pentosides found in low PhP-V emitters will generally be referred to as ‘triglycosides’.

**The pool of glycosidically-bound phenylpropanoid volatiles has the capacity to account for the volatiles produced upon fruit tissue disruption**

It is well known that some tomato volatiles accumulate in fruit tissue during ripening and are stored as non-volatile sugar conjugates (Buttery et al., 1990; Marlatt et al., 1992; Ortiz-Serrano and Gil, 2007). It has been suggested that the cleavage of such glycosidically bound volatile precursors is mediated through glycosidasces liberated or activated upon disruption of cellular compartments when cells become stressed or are damaged (Mizutani et al., 2002). To determine the potential contribution of PhP volatile glycosides to the emission of PhP volatiles upon fruit tissue disruption (blending), we quantified the amounts of glycosidically bound PhP volatiles in tomato fruit by measuring the amounts of PhP volatiles emitted from a crude extract specifically enriched for glycosides, after treatment with a carbohydrase enzyme preparation possessing a broad spectrum of glycosylhydrolytic activity. To relate these volatile levels to those released upon fruit tissue blending, levels of guaiacol, methyl salicylate and eugenol emitted from 49 high PhP-V fruits of the 94 cultivar collection were quantified using authentic chemical standards. The variation in the levels of guaiacol, methyl salicylate and eugenol emitted within 10 minutes after tomato fruit blending is shown in Figure 2A.

Crude glycosidic extracts were prepared from equal starting amounts of bulked fruit samples of 6 low and 6 high PhP-V cultivars and subjected to complete glycoside hydrolysis using Viscozyme L – a crude carbohydrase preparation derived from *Aspergillus* sp.. Volatiles emitted upon the hydrolysis were analyzed by SPME-GC-MS and amounts of guaiacol, methyl salicylate and eugenol were quantified using authentic standards of these compounds, as described in the Materials and Methods. The results show that glycosidic extracts of both high and low PhP fruits have a similar capacity to release PhP-derived volatiles upon cleavage by glycosidases (Figure 2B). Furthermore, the levels of PhP volatiles released from these glycosidic extracts exceeded the amounts of PhP volatiles emitted from high PhP fruits within 10 minutes after blending. We calculated that the high PhP-V fruit extracts used consisted of 0.096 ± 0.014 ppm of guaiacol, 0.14 ± 0.008 ppm of methyl salicylate and 0.67 ± 0.09 ppm of eugenol, present as bound as glycososides. Low PhP-V fruit extracts consisted of 0.066 ± 0.014 ppm of bound guaiacol, 0.098 ± 0.009 ppm of bound MeSA and 0.53 ± 0.034 ppm of bound eugenol (Figure 2B). The increase in volatiles released upon glycosidase treatment, both in high and low PhP-V glycosidic extracts, was accompanied by a complete hydrolysis of the corresponding PhP-V
glycosides (Figure 2C, D). These results suggest that the pool of glycosidically bound PhP volatiles has the capacity to account for all the volatiles released upon fruit blending. However, despite the presence of sufficient amounts of glycosidically bound PhP volatiles in low PhP-V fruits, these fruits do not release any significant amounts of PhP volatiles upon blending without the need for additional synthesis.

It was not possible to quantify the PhP-V glycosides directly due to a lack of authentic standards. The viscozyme-mediated hydrolysis, however, enabled us to quantify the amounts of PhP-V glycosides as amounts of volatiles bound to glycosides, by measuring the total amount of volatiles released after complete hydrolysis of the glycosidic extracts.

**Cleavage of PhP-V glycosides upon fruit tissue disruption is restricted to high PhP-V fruits**

To investigate whether the release of PHP volatiles upon fruit blending was due to endogenous hydrolysis of their corresponding diglycosides, we analyzed the dynamics of PhP volatile emission. Frozen powder of red fruits from 6 high and 6 low PhP-V genotypes was incubated for 0, 3, 6, 12 and 24 minutes at 30°C. At each time point, levels of both volatile and non-volatile compounds were measured using SPME-GC-MS and LC-QTOF-MS, respectively. In the high PhP-V genotypes emission of all three PhP volatiles increased in time (Figure 3A, B, C). For guaiacol and eugenol, emission increased continuously over the 24 minute period while the amount of methyl salicylate released into the headspace peaked at 12 min and then decreased (Figure 3B). Concomitant with the increase in the three volatiles, the levels of their diglycosides decreased. The amounts of guaiacol and eugenol emitted were comparable to the calculated amounts of the corresponding diglycosides cleaved at each of the time points after fruit blending (Figure 3A, C). However, emission of methyl salicylate was lower than expected based on the amount of methyl salicylate diglycoside cleaved (Figure 3B). This might be due to a process acting in parallel to the methyl salicylate release, for example by conversion or reduction of methyl salicylate before it is emitted into the headspace.

Throughout the experiment, no detectable emission of the three PhP volatiles could be observed from fruit material of the low PhP genotypes (results not shown). In addition, the abundance of all low PhP-V triglycoside species remained constant over the 24 minutes period after fruit blending (Figure 4), indicating that their cleavage did not occur. This is in contrast, as described above, to the case of high PhP-V cultivars where this incubation period was clearly sufficient to release enough PhP volatiles from their corresponding diglycosides to account for the total amount of volatiles produced (Figure 3). Low PhP-V fruits therefore lack the capacity to emit guaiacol, methyl salicylate and eugenol from their corresponding triglycosides upon fruit tissue disruption.
PhP-V glycosides identified in low PhP-V fruits are resistant to endogenous hydrolysis upon fruit tissue disruption

The observations above may be due either to differences in glycosidase activity of high and low PhP-V fruit matrix or to differences in susceptibility of the high and low PhP-V glycoside species to endogenous glycosidases. To investigate this, low and high PhP-V fruit materials (bulks of fruits of the 6 low PhP-V and the 6 high PhP-V cultivars used for experiments described above) were spiked with glycosidic extracts containing different PhP glycosides, in a reciprocal way. Firstly, we studied whether the low PhP-V fruit matrix has the capacity to hydrolyse PhP-V diglycosides similar to the high PhP-V fruits they were derived from. For this, a crude glycosydic extract derived from high PhP-V fruits and previously used for the quantitative analysis of bound PhP volatiles (Figure 2B), containing diglycosides of guaiacol, MeSA and eugenol, was added to (a) blended low PhP-V fruits and (b) to blended high PhP-V fruits as a control. LC-MS analysis of spiked and non-spiked tomato matrix revealed that the following amounts of PhP-V diglycosides were spiked in: 0.40 ± 0.023 ppm of guaiacol, 0.63 ± 0.018 ppm of MeSA and 4.28 ± 0.26 ppm of eugenol. The spiked fruit samples were incubated for 10 minutes at 30°C as described before. Spiking of high PhP-V fruits with a high PhP-V glycosidic extract containing PhP-V diglycosides resulted in significant increases in the emission of the PhP volatiles guaiacol ($\Delta$emission = 0.106 ± 0.01 ppm, $p < 0.01$ - significance of difference compared to the non-spiked control), methyl salicylate ($\Delta$emission = 0.094 ± 0.007 ppm, $p < 0.001$) and eugenol ($\Delta$emission = 0.69 ± 0.014 ppm, $p < 0.001$) compared to the amounts of these volatiles emitted from non-spiked high PhP-V fruit material (Figure 5A). The amounts of diglycosides decreased accordingly, except for methyl salicylate, which was emitted at a lower amount than was anticipated based on the hydrolysis of the corresponding diglycoside. A comparable increase in the emission of PhP volatiles was found when low PhP-V fruit matrix was spiked with a high PhP-V glycosidic extract (Figure 5B). These results indicate that low PhP-V fruits have a glycosylhydrolytic activity similar to high PhP-V fruits and suggest that the difference in PhP volatile emission observed between fruits of the two groups of tomatoes is due to resistance of low PhP-V glycosides to endogenous hydrolysis rather than to differences in glycosidase activity.

To study this hypothesis, the high PhP-V fruit matrix was spiked with a crude low PhP glycosidic extract containing triglycosides. Based on the proportion of diglycoside and triglycoside content in high and low PhP-V glycosidic extracts, respectively (Figure 2B), we calculated that the following additional amounts of PhP-V triglycosides were present in spiked samples: 0.28 ± 0.05 ppm of bound guaiacol, 0.44 ± 0.04 ppm of bound MeSA and 3.38 ± 0.23 ppm of bound eugenol. None of the spiked guaiacol, methylsalicylate and eugenol triglycosides were cleaved (Figure...
6A), confirming that these triglycosidic forms are resistant to hydrolysis by the endogenous glycosidases in both low and high PhP-V fruits. Indeed, spiking with low PhP-V glycosides did not lead to any significant increase in the emission of guaiacol and eugenol compared to the non-spiked control (Figure 6B) and the levels of these compounds could well be explained by the amount of the corresponding diglycosides cleaved (Figure 6C). However, rather than increased, emission of both guaiacol and eugenol tended to be decreased relative to the non-spiked control (Figure 6B). For eugenol this decrease appeared to be significant (p < 0.001). This was consistent with a decrease in hydrolysis of the corresponding diglycosides (Figure 6C). The observed decrease in diglycoside hydrolysis / volatile emission suggests that the activity of the endogenous glycosyl hydrolase is inhibited by the crude low PhP glycosidic extract. One could speculate that the excess of PhP triglycosides added inhibits the glycosyl hydrolase activity, for example by competing with the endogenous diglycosides for binding to the active site of the glycosyl hydrolase enzyme.

Also the cleavage of methylsalicylate diglycoside was inhibited by the low PhP glycosidic extract, but the amounts cleaved could well account for the amount of methylsalicylate released (Figure 6B, C). Surprisingly, however, the emission of methylsalicylate did not follow the pattern of the diglycoside cleavage, since it slightly increased in spiked samples, rather than decreased (Figure 6B). This apparent discrepancy is most likely due to an effect of the crude low PhP glycosidic extract on processes that may be acting in parallel to the methylsalicylate release, as observed in the time-course experiments (Figure 3B).

**The PhP volatile emission contrast develops during fruit ripening**

Evidently, the difference in PhP volatile emission between ripe fruits from low and high PhP-V tomato cultivars is related to the difference in their glycosylation patterns. To investigate whether fruit development plays a role in establishing the observed difference in PhP volatile glycosylation, we studied the dynamics of both PhP volatile emission and glycoside accumulation during normal fruit ripening. Fruits of 3 low and the 3 high PhP-V genotypes were harvested at mature green, breaker, turning and red ripe stages. The emission of PhP volatiles after blending was analyzed by SPME-GC-MS and the amounts of the corresponding glycosides in the intact fruit were determined by LC-QTOF-MS. In fruits of high PhP-V genotypes, the levels of the PhP volatiles guaiacol and methyl salicylate emitted at mature green, breaker and turning stages were found to be similar to the levels observed in red ripe fruits of these genotypes (Figure 7A, B). The emission of eugenol from mature green fruits was low, but increased markedly upon fruit maturation (Figure 7C). The abundance of all three PhP-V diglycosides in intact fruits showed patterns similar to those of the volatile aglycones (Figure 7D, E, F). Mature green fruits of the low
PhP-V cultivars emitted amounts of guaiacol, methyl salicylate and eugenol that were comparable with those observed in green fruits of high PhP-V genotypes (Figure 7A, B, C). This was also observed for the corresponding diglycosides, except for eugenol diglycoside, which could not be detected in low PhP-V fruits at any of the ripening stages (Figure 7D, E, F). During maturation of low PhP-V fruits, levels of both the PhP volatiles and the corresponding diglycosides declined to barely detectable levels at turning and red ripe stages. However, this decline was accompanied by an increase in the levels of all PhP-V triglycosides (Figure 7G, H, I). These results clearly show that, in fruits of high PhP-V cultivars, PhP volatiles can be emitted at all stages of ripening. However, low PhP-V lines only have this capacity at the mature green stage. This suggests that a developmentally-regulated program, switched either on or off at the breaker stage, determines the emission of phenylpropanoid volatiles in fruits of high versus low PhP-V cultivars, by modifying the chemical structure of their non-volatile glycosidic precursors. The conversion from a diglycoside into a (malonyl)triglycoside likely prevents the glycosidic bonding being cleaved by endogenous enzymes. Consequently, aglycone release upon tissue disruption is prevented, thus leading to the low PhP-V phenotype in red fruit (Figure 8). This suggests a novel concept for the regulation of PhP volatile production, not by activating or deactivating the volatile biosynthetic pathway, but by changing the glycoconjugate structure of the volatile precursors in question, thereby making them unavailable to subsequent enzymatic hydrolysis upon decompartmentalization.

Discussion

_Emission of PhP volatiles from tomato fruit upon tissue disruption is due to cleavage of the corresponding hexose-pentosides_

We have previously shown that polyphenol compounds in general play a significant role in determining the phenotypic and biochemical differences between tomato tissues and genotypes (Bovy et al., 2007; Moco et al., 2007; Butelli et al., 2008; Schijlen et al., 2008; Bovy et al., 2009). The production of phenylpropanoid-derived volatiles (PhP-V), such as methyl salicylate, guaiacol and eugenol, was also found to be one of the most important determinants of the metabolic
variation in a set of 94 tomato cultivars, chosen to represent the current commercial tomato
germplasm. The ability to emit these volatiles upon fruit tissue disruption divided the genotype set
into two groups, irrespective of their physical phenotype (Tikunov et al., 2005).

Analysis of complex metabolomics data reveal that correlating compounds are often
biochemically related or they can be linked as precursor-product in a biochemical pathway
(Ursem et al., 2008; Gavai et al., 2009). In the present study we aimed to unravel the biochemical
mechanisms underlying the observed difference in PhP volatile emission, by searching for causal
relationships between volatiles and their potential biochemical precursors – non-volatile
compounds. Red-ripe fruits of 94 tomato cultivars were therefore profiled using SPME-GC-MS to
detect volatiles and LC-QTOF-MS to detect semi-polar, non-volatile metabolites. Data fusion
strategies using GC-MS and LC-MS datasets, followed by multivariate analysis, revealed a
genotype structure which was mostly determined by the PhP volatiles and a number of non-
volatile metabolites. Those non-volatile metabolites which correlated positively to the PhP
volatiles were putatively identified as being the diglycosides of these volatiles, consisting of a
hexose-pentose moiety as determined by LC-MS and -MS/MS accurate mass fragmentation
patterns. These analytical tools used, however, do not allow for discrimination between different
pentose and hexose sugar isomers. Several hexose-pentose combinations, ubiquitous in nature,
can potentially be attached to these volatile aglycones, producing different types of diglycosides,
e.g. β-L-arabinofuranosyl-β-D-glucopyranosid, β-D-apiofuranosyl-β-D-glucopyranoside
(vicianoside), β-D-xylopyranoyl-β-D-glucopyranoside (primeveroside) (Crouzet and Chassagne,
1999).

Many volatile compounds are detectable as glycoconjugates in tomato fruit (Buttery et al.,
1990; Marlatt et al., 1992; Ortiz-Serrano and Gil, 2007). Emission of the three phenylpropanoid
volatiles studied here has been observed upon enzymatic or acid hydrolysis of tomato fruit
glycosidic extracts (Marlatt et al., 1992; Ortiz-Serrano and Gil, 2007). In vivo, volatile compounds
can be liberated from their glycosidically bound forms e.g. upon plant cell disruption during
herbivore attack, fruit processing or consumption. Cell disruption leads to mixing of subcellular
compartments and, thus, volatile glycosides become exposed to endo- or exogenous cleavage
enzymes – such as glycosyl hydrolases (glycosidases). Our results suggest that the quick and
intense emission of PhP volatiles occurring upon fruit tissue disruption (blending) can be
explained by cleavage of the corresponding diglycosides by endogenous glycosidase(s), since (i)
the tomato fruit tissue disruption time course experiment (Figure 3) showed that the increase of
PhP volatile emission was accompanied by a quantitatively comparable reduction of the
corresponding diglycosides, (ii) the in vitro hydrolysis of crude tomato fruit glycosides mediated by
a carbohydrase preparation showed that the pool of glycosidically-bound phenylpropanoid
volatiles has the capacity to account for the volatiles produced upon tissue grinding and (iii)
spiking blended tomato fruit matrix with a crude glycosidic extract derived from high PhP-V fruit
material, containing PhP-V diglycosides, resulted in enhanced emission of PhP volatiles, not only when spiked in the high PhP-V fruit matrix, but also upon spiking into the low PhP-V fruit matrix. The latter result indicates that both high and low PhP-V tomato fruits have a similar capacity to cleave PhP-V diglycosides upon tissue disruption, but that volatile emission is determined by the presence of these specific volatile diglycosides in the tomato fruit.

Interestingly, in the tomato fruit blending time series experiment, the amount of methylsalicylate emitted into the headspace increased in time until a maximum emission was reached at approximately 10 minutes of incubation, and decreased again at longer incubation times. At the same time, the amount of the corresponding hexose-pentoside was nevertheless continuously reduced over the entire 24 min period. We hypothesize that this decrease is due to a process which acts in parallel to the release of methyl salisylate and causes endogenous loss of methyl salicylate, e.g. through its conversion into the non-volatile salicylic acid. This process has already been described for methylsalicylate in relation to its airborne signaling ability (Shulaev et al., 1997). These authors demonstrated that methyl salicylate could be transmitted to neighboring plants and subsequently induce their defensive mechanisms by converting methylsalicylate into salicylic acid. An enzyme SABP2 (Salicylic Acid Binding Protein) mediating the conversion of methyl salicylate into salicylic acid has been identified (Forouhar et al., 2005). Thus MeSA acts as mobile signal molecule in systemic acquired resistance (Park et al., 2007). If this well known process also takes place in tomato fruit, our experiments demonstrate the existence of a mechanism to initiate such communication through a quick release of considerable amounts of methyl salicylate and the other two phenylpropanoid volatiles guaiacol and eugenol, by cleavage of their corresponding diglycosides upon tissue damage/disruption. This process may be of particular importance in leaves, where we could detect all three PhP-V hexose-pentosides in both high and low fruit PhP-V genotypes (data not shown).

**PhP volatile emission is arrested in low PhP-V fruits, due to a ripening-induced modification of the glycosylation pattern**

Emission of PhP volatiles upon tissue disruption, by cleavage of the corresponding diglycosides, was observed in fruits of only a proportion of the cultivars tested. We hypothesized that in fruits which did not emit PhP volatiles, these compounds were present in a different non-cleavable form. Indeed, when fusing GC-MS and LC-MS datasets we identified non-volatile metabolites that accumulated in low PhP-V fruits and which were inversely correlated to the presence of PhP volatiles and their non-volatile diglycoside precursors. Later, GC-MS and LC-MS analysis of crude glycoside extracts hydrolyzed *in vitro* using a fungal crude carbohydrate preparation revealed that the amount of PhP volatiles emitted from crude glycosides extracted from low PhP-V
V fruits was comparable to the amount of these volatiles emitted from high PhP-V glycoside extracts (Figure 2B). These observations led to the hypothesis that in low PhP-V fruits, PhP volatiles were present but in a different conjugated form. Essentially, in low PhP fruits, conjugation goes a step further during ripening. The new conjugated forms could be cleaved with the carbohydrate preparation in vitro to emit PhP volatiles, but not by endogenous carbohydrate naturally present in tomato fruits. Using LC-MS/MS analyses we found that in the low PhP-V fruits methyl salicylate, guaiacol and eugenol are present as various glycoside species different from the hexose-pentosides present in high PhP-V fruits. Analysis of MS/MS spectra of these glycosides revealed two major sugar moieties conjugated to guaiacol, MeSA and eugenol: (i) a dihexose-pentose moiety and (ii) a malonyl dihexose-pentose moiety. Triglycosides of volatiles consisting of different sugar combinations have been reported in plants before (Herderich et al., 1992; Bilai et al., 1994; Kijima et al., 1997). Also, malonylation of sugar moieties is a common feature in several plants (Withopf et al., 1997; Kazuma et al., 2003; D’Auria et al., 2007; Kogawa et al., 2007). To our knowledge, however, malonylated triglycosides of volatile compounds have not been described in plants to date. Malonylation of secondary metabolite glycosides might play a role in enhancing metabolite solubility, resistance to glycosidase-driven cleavage and differential targeting of organic compounds to either the vacuole or the cell wall (Day and Saunders, 2004; Dhaubhadel et al., 2008). Our results, showing that neither dihexose-pentosides of PhP volatiles nor their malonylated forms could be cleaved by endogenous glycosylhydrolases induced upon disruption of both low and high PhP-V fruits, indicate that the addition of the second hexose rather than the malonylation is the primary factor determining the resistance of the low PhP-V triglycoside species to endogenous hydrolysis. The additional malonylation might serve other functional purposes.

Unripe mature green fruits of both low and high PhP-V emitters (at red-ripe stage) revealed the presence of comparable amounts of PhP-V diglycosides. As a result, both types of fruit did not reveal a significant difference in PhP volatile emission at this ripening stage. Along with the results of the in vitro enzymatic hydrolysis of red-ripe fruit glycosidic extracts these results suggest that the biosynthetic pathways leading to the production of the three phenylpropanoid volatiles are equally active in both types of fruit and have the capacity to produce comparable amounts of these volatiles throughout fruit ripening. The difference in emission of PhP volatiles upon fruit blending develops as fruit ripening progresses and results from the ripening-dependent additional modification of the glycosylation patterns of these volatiles. In low PhP-V fruits, the cleavable PhP-V diglycoside species are converted into non-cleavable triglycoside species from breaker stage onwards. The addition of the second hexose onto the first hexose of the diglycoside, which is obvious from the MS/MS fragmentation (Table 1) where dihexose fragments could be found, prevents hydrolysis of the resulting triglycosides upon fruit blending (Figure 8).
At this moment we can only speculate on which genes and/or enzymes are involved in the synthesis of the PhP-V triglycosides. Furthermore, we also have no clear view as to their genetic origin or to how their distribution relates to parentage. Likely the enzymes belong to two classes: (i) glycosyl transferases (GTs), which transfer a nucleotide diphosphate-activated sugar group to an aglycone or an already existing sugar moiety and (ii) malonyl transferases, transferring a malonyl group from malony-CoA to a sugar moiety. Approximately 15 malonyltransferases have been described in the plant kingdom to date (D'Auria, 2006; Suzuki et al., 2007; Unno et al., 2007). More than 100 GTs with a glycosylation activity for small molecules have been described in *Arabidopsis* (Bowles et al., 2005). Functional characteristics and substrate specificities of many of these genes are not completely known. Preliminary results of our own gene expression experiments revealed approximately 100 GT genes expressed in tomato fruit (data not shown). We are currently employing various strategies (genetic, expression and functional gene analyses) to find those GT genes involved in the PhP-V glycoside modification. In summary, we showed that, in tomato fruit, emission of the phenylpropanoid volatiles guaiacol, methyl salicylate and eugenol appear to be regulated by means of a developmentally-programmed modification of their glycosilated precursors. Tomato fruits have a large reserve of important flavor volatiles stored as glycosides (Buttery et al., 1990; Marlatt et al., 1992; Ortiz-Serrano and Gil, 2007). This principle influencing the emission of flavor volatiles through their glycoconjugate modification could pave the way for new strategies to control fruit quality characteristics such as flavor and taste. Besides fruit quality aspects, this biochemical process may also play an important role in plant-environment interactions, including e.g. the response to biotic stresses. At this moment it is not completely clear how this mechanism has evolved. It could have appeared as a by-product of extensive tomato breeding activities that now can be considered as a potentially beneficial trait. On the other hand, considering that fruit flavour is an important characteristic for natural seed-dispersing organisms (Goff and Klee, 2006; Schwab et al., 2008) one can assume that this mechanism could have already evolved in wild tomato germplasm. Indeed, we have preliminary data suggesting that there is variation for this trait in wild tomato germplasm (data not shown). Nevertheless this question will require a further investigation. A combination of fusion of data derived from a modern multi-instrumental metabolic profiling platform and a classical quantitative analysis of biochemical processes, appears to be a powerful approach to elucidate the biochemical principle underlying phenylpropanoid volatile emission in tomato fruit. In addition, many other volatile-non volatile metabolite interactions could be observed. Our current activities are aimed at unraveling the functional significance of a broader range of these interactions.
Materials and Methods

Plant material

Seeds from 94 tomato (*Solanum lycopersicum* L.) cultivars were obtained from 6 different tomato seed companies, each having its own breeding program. As such, the cultivars should represent a considerable collection of genetic and therefore phenotypic variation, not just between tomato types (cherry, round and beef), but also within the individual genotypes of each fruit type. This study was initially performed ‘blind’ and the only information received from the company breeders concerned fruit type. No information was supplied on their genetic background. For fruit type classification, breeders generally use a combination of (i) fruit diameter and (ii) number of locules in the fruit (fl). For the latter, the criteria were: cherry type fl = 2; round fl = 3, beef fl ≥ 4. Two independent experiments were performed over two seasons: one in 2003 and one in 2004. A study of the volatile compounds from ripe fruits of plants, specifically from the 2003 experiment, has been described before (Tikunov et al., 2005). The present study was performed on the second experiment carried out in 2004. As in 2003, all cultivars were grown in the summer under greenhouse conditions at a single location in Wageningen, The Netherlands; 9 plants, randomly distributed over 3 adjacent greenhouse compartments were grown for each cultivar, giving a total of ca 850 individuals. Pink-staged tomato fruits were picked from all plants on two consecutive days. To mimic the conditions “from farm to fork”, fruits were then stored for 1 week at 15°C followed by one day at 20°C prior to sampling and freezing in liquid nitrogen. During this 8d period, the fruits continued to ripen slowly and, at the moment of sampling, were fully red-ripe, resembling the situation at the time of consumption. In addition, fruits of each of the 94 cultivars were collected at 3 developmental stages: mature green, breaker and turning. These stages were judged according to a standardized fruit color scheme provided by The Greenery (Valstar Holland b.v., Poeldijk, The Netherlands). To make a representative fruit sample, for each cultivar, and at each of the 4 ripening stages, a number of identical red-ripe fruits was pooled; 12 for round and beef tomatoes and 18 for cherry tomatoes. The fruit material was immediately frozen in liquid nitrogen, ground in an analytical electric grinder and stored at -80°C until analysis.

SPME-GC-MS profiling of tomato fruit volatile organic compounds (VOC)

The profiling of volatile metabolites was performed using a headspace SPME-GC-MS method (Tikunov et al., 2005; Tikunov et al., 2007). Frozen fruit powder (1 g fresh weight) was weighed into a 5-ml screw-cap vial, closed and incubated at 30°C for 10 minutes. An aqueous EDTA-NaOH solution was prepared by adjusting 100 mM EDTA to pH of 7.5 with NaOH. Then, 1 mL of the EDTA-NaOH solution was added to the sample to give a final EDTA concentration of 50 mM. Solid CaCl₂ was then immediately added to give
a final concentration of 5 M. The closed vials were then sonicated for 5 minutes. A 1 mL aliquot of the pulp was transferred into a 10-mL crimp cap vial (Waters), capped and used for SPME-GC-MS analysis.

Volatile were automatically extracted from the vial headspace and injected into the GC-MS via a Combi PAL autosampler (CTC Analytics AG). Headspace volatiles were extracted by exposing a 65 μm PDMS-DVB SPME fiber (Supelco) to the vial headspace for 20 minutes under continuous agitation and heating at 50°C. The fiber was desorbed in a GC 8000 (Fisons Instruments) injection port 1 min at 250°C. Chromatography was performed on an HP-5 (50m x 0.32 mm x 1.05 μm) column with helium as carrier gas (37 kPa). The GC interface and MS source temperatures were 260°C and 250°C, respectively. The GC temperature program began at 45°C (2 min), was then raised to 250°C at a rate of 5°C min⁻¹ and finally held at 250°C for 5 min. The total run time including oven cooling was 60 min. Mass spectra in the 35 – 400 m/z range were recorded by an MD800 electron impact MS (Fisons Instruments) at a scanning speed of 2.8 scans/sec and an ionization energy of 70 eV. The chromatography and spectral data were evaluated using “Xcalibur™” software (http://www.thermo.com).

LC-QTOF-MS, MS/MS analysis of semi polar tomato fruit compounds

The extraction and the LC-QTOF-MS analysis of semi-polar compounds were performed according to the protocol described in (Moco et al., 2006); 0.5 g frozen tomato fruit powder (FW) of each of the 94 cultivars was extracted with 1.5 mL formic acid:methanol (1:1000, v/v) solution. The extracts were sonicated for 15 min and filtered through a 0.2 μm inorganic membrane filter (Anotop 10, Whatman).

An LC-QTOF-MS platform was used for the profiling of the extracts. This platform consisted of a Waters Alliance 2795 HT HPLC system equipped with a Luna C18(2) pre-column (2.0 x 4 mm) and an analytical column (2.0 x 150 mm, 100 Å, particle size 3 μm; Phenomenex, Torrance, CA, USA) connected to an Ultima V4.00.00 QTOF mass spectrometer (Waters-Corporation, MS technologies, Manchester, UK). Degassed solutions of formic acid:ultra pure water (1:1000, v/v – eluent A) and formic acid:acetonitrile (1:1000, v/v – eluent B) were pumped into the HPLC system at 190 µL min⁻¹ and the gradient linearly increased from 5% to 35% eluent B over a 45 min period, followed by 15 min of washing and equilibration of the column. The column, sample and room temperatures were kept at 40, 20 and 20°C, respectively.

Ionization was performed using an electrospray ionization (ESI) source and masses were detected in negative mode. A collision energy of 10 eV was used for full-scan LC-MS in the range of m/z 100-1500. For LC-MS/MS, increasing collision energies of 10, 15, 25, 35 and 50 eV were applied. Leucine enkephalin, [M – H]⁻ = 554.2620, was used for on-line mass calibration (lock mass).

Preparation of tomato fruit extracts enriched with crude glycosides
Two bulked tomato fruit samples, high PhP-V and low PhP-V, were prepared by mixing equal amounts of 6 high PhP-V and 6 low PhP-V genotypes, respectively. Methanolic extracts were prepared by the extraction of 40 g FW of each of the bulks in 120 ml 100% methanol with 1 hour agitation at room temperature. The methanol was then removed from the supernatant in a vacuum rotary evaporator at 40°C and the glycoside residue was re-dissolved in 50 ml pure water. The extract was passed through a glass column (35 x 1 cm I.D.) packed with up to 20 cm Amberlite XAD-2 resin (Supelco Inc., Bellefonte, USA). The flow rate used was 2 mL min⁻¹. The column was then rinsed with 50 mL water, followed by 50 mL hexane. Bound compounds were then eluted from the column using 50 ml methanol which was then evaporated under vacuum at 40°C.

Quantitative analysis of glycosidically bound PhP volatiles

A lack of authentic chemical standards did not allow direct quantification of PhP-V glycosides identified in the present study. Authentic chemical standards of guaiacol, methyl salicylate and eugenol are available (Sigma-Aldrich). Thus the amounts of PhP-V glycosides, measured as LC-MS detector response of a parent molecular ion, were expressed as amounts of corresponding volatiles (in ppm) released upon their complete hydrolysis. For this, dilution series of crude glycosides extracted from both low and high PhP-V fruits were prepared and subjected to enzymatic hydrolysis and amounts of glycosides present and volatiles released were measured by LC-QTOF-MS and SPME-GC-MS, respectively. Firstly, aliquots of a glycosidic extract corresponding each to 16 g FW original fruit tissue material of low and high PhP-V fruits were re-dissolved in 1.5 mL of phosphate-citrate buffer (0.2 M, pH 5.4). The solution obtained was divided into two series of five aliquots of 50, 100, 150, 200 and 250 μL. The first aliquot series was used to estimate original amounts of PhP-V glycosides present. For this, each of the aliquots was adjusted to 1 mL with phosphate-citrate buffer and 3 mL of MeOH was added. The solutions obtained were analyzed by LC-QTOF-MS. One of the two aliquot series was placed in 1.5 mL screw-cap vials, 200 μL of Viscozyme L (Sigma-Aldrich) – a carbohydrase preparation, derived from Aspergillus sp., was added to each of the aliquots as a hydrolytic agent and the total volume of each of the samples was adjusted to 1 mL with the phosphate-citrate buffer. Vials were closed and incubated at 40°C for 72 hours. After the incubation 0.5 mL of each sample was extracted with 1.5 mL of MeOH and subjected to LC-QTOF-MS analysis as described above to ensure complete hydrolysis of PhP-V diglycosides. The remaining 0.5 mL of each of the hydrolyzed samples was mixed with 0.5 mL of NaOH-EDTA mixture (100 mM, pH 7.5) and solid CaCl₂ 5M final concentration in a 10 mL headspace vials. These samples were subjected to SPME-GC-MS analysis of volatile compounds produced upon glycoside hydrolysis. Amounts of guaiacol, methyl...
salicylate and eugenol emitted after hydrolysis of glycosides were quantified using calibration curves. For this purpose different amounts of authentic standards of these volatiles were diluted in the medium identical to the medium used for the enzymatic hydrolysis and analyzed by SPME-GC-MS. The following concentration ranges of volatiles were used: 0.1 – 0.5 ppm of guaiacol and methyl salicylate and 0.05 – 0.25 ppm of eugenol. The calculated amounts of bound volatiles in crude glycosidic extracts, determined through their release after viscozyme treatment, were expressed as ppm present in the original high- and low PhP tomato fruits (Figure 2B).

As shown in the Result section, in high PhP-V fruits guaiacol, MeSA and eugenol exist as a single major glycoconjugated form – diglycosides (hexose-pentosides). Thus, the amounts of PhP-V diglycosides could be expressed as ppm using linear equations derived by fitting the diglycoside LC-MS responses of non-hydrolyzed glycoside aliquot series to quantitative data of volatiles released upon their complete hydrolysis achieved in 72 hours: \( y = a + b \cdot x \), where \( y \) - a diglycoside MS detector response, \( x \) - an amount of a corresponding volatile released upon the complete diglycoside hydrolysis in ppm, \( a \) - an intercept set at 0, \( b \) - a coefficient that is equal to: for guaiacol: \( 0.00431 \pm 3e-4 \), methyl salicylate: \( 0.00285 \pm 5e-5 \) and eugenol: \( 0.00329 \pm 2.7e-4 \). These equations were used to quantify amounts of diglycosides in all experiments of the present study.

Individual quantification of the PhP-V triglycosides present in the low PhP-V fruits was not possible, since they exist in two major forms: dihexose-pentosides and malonyl-dihexose-pentosides, which can have different ionization efficiencies. Therefore, the amounts of the PhP-V triglycosides in the fruit tissue disruption series, the spiking experiments and in the analysis of fruit ripening stages were expressed relative (%) to their levels in intact red ripe fruits. The total amount of bound volatiles that both triglycoside forms account for could be calculated based on the viscozyme-mediated hydrolysis, as described above.

Analysis of PhP-V emission and PhP-V glycoside hydrolysis upon tomato fruit disruption

Aliquots of 1 g FW red ripe fruit material of each of the cultivars selected for the experiment were incubated in duplicate in closed 5-mL vials for 0, 3, 6, 12, 24 minutes at 30°C. For the first series of aliquots, the incubations were terminated at each time point by addition of a CaCl₂/EDTA mixture as described above and the resulting mixtures were analyzed for volatiles using SPME-GC-MS. The duplicate series of aliquots was extracted with 3 mL formic acid:methanol (1:1000, v/v) solution and subsequently analyzed for PhP volatile glycosides using LC-QTOF-MS as described in the corresponding sections above.
GC-MS data processing

The 94 VOC profiles derived using the SPME-GC-MS method were processed by the MetAlign™ software package (www.metalign.nl) for baseline correction, noise estimation and ion-wise mass spectral alignment. The data matrix obtained was subjected to a fragment ion clustering for data size reduction and putative compound mass spectra extraction using the Multivariate Mass Spectral Reconstruction (MMSR) approach (Tikunov et al., 2005). The MMSR procedure was performed using a C++ based software package which was developed in-house. Each cluster in the reduced dataset was represented by a single ion fragment, i.e. the most abundant fragment ion that reflected an average intensity pattern of an entire cluster (putative compound mass spectrum) derived by MMSR. The mass spectra of the clusters derived (number of fragment ions in a mass spectrum ≥ 5) were then subjected to a tentative identification using the NIST mass spectral library (www.nist.gov).

LC-QTOF-MS data processing

Like the GC-MS profiles, the profiles derived by LC-QTOF-MS were processed by MetAlign™ software (at settings as described in Moco et al. (2006)) and the dataset obtained was reduced using the MMSR approach. Similar to GC-MS data, the reduced LC-MS data consisted of single masses representative of an ion cluster (including isotopes, adducts and fragments obtained by unintended in-source fragmentation). Also ion fragments not correlating to any cluster by MMSR and with a maximum intensity more than 200 counts/scan (a threshold for a reliable accurate mass calculation with our QTOF instrument) were manually added to the data matrix.

Data normalization, fusion and multivariate analysis

Prior to fusion of the two data sets, the ion fragment intensities were normalized using log2 transformation and standardized using range scaling, in which each value in a certain row, corresponding to a specific ion, was divided by the intensity ranged observed for this row throughout all samples analyzed (Smilde et al., 2005). Each row was then mean centered. Finally, both data matrices were concatenated along sample (genotype) dimension yielding a fused data matrix with normalized and log-transformed signal values. Principal Components Analysis (PCA) implemented in GeneMath XT version 1.6 software (www.applied-maths.com) was used for unsupervised cluster analysis of the metabolites. Pearson’s product-moment correlation coefficient was used as a measure for metabolite-metabolite correlation.
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Legends

Figure 1. Principal Component Analysis of the LC-MS/GC-MS fused data obtained from 94 contrasting tomato genotypes.

A - clustering of tomato genotypes along the first 3 principal components (PC): PC1, PC2 and PC3 describe 17.6%, 16.4% and 9.0% of the total metabolic variation in fruits of the 94 tomato cultivars.

B – clustering of volatile and non-volatile compounds determining the genotype structure shown in panel A.

Fruit type-independent clustering of genotypes (panel A, encircled red and green) along PC2 was determined by the three groups of compounds shown in panel B: one group of volatile compounds detected by GC-MS (GC1), consisting of the PhP volatiles methyl salicylate, guaiacol and eugenol (GC1 group) and two groups of non-volatile compounds detected by LC-MS: LC1 and LC2. The GC1 and LC1 compounds showed a high abundance in high PhP-volatile (PhP-V) genotypes (panel A, encircled red) and LC2 compounds showed higher abundance in low PhP-V genotypes (panel A, encircled green).

Figure 2. Amounts (ppm: mg/kg FW) of guaiacol, methyl salicylate and eugenol emitted (A) from blended fruits of 49 high PhP-V cultivars upon fruit blending (10 min incubation at 30°C) and (B) upon complete viscozyme-mediated in vitro hydrolysis of crude glycoside extracts derived from high and low PhP-V fruits. Both PhP-V diglycosides (C) and triglycosides (D) were completely hydrolyzed after 72 hours of viscozyme-mediated hydrolysis.
Figure 3. Dynamics of PhP volatile production upon fruit cell disruption. Ground and frozen powder of red fruits from 6 high PhP-volatile (PhP-V) genotypes was incubated for 0, 3, 6, 12 and 24 minutes at 30°C (which included thawing). At each time point, levels of both volatiles and non-volatile compounds were measured using SPME-GC-MS and LC-QTOF-MS, respectively. Average patterns (+/- SD) of 6 high PhP-V genotypes are presented. A, B and C – emission of guaiacol, methyl salicylate and eugenol and decrease of corresponding hexose-pentosides (HP), respectively. Red broken lines (●) represent amounts of the volatiles emitted at each of the time points, solid blue lines (■) represent the calculated absolute amount of corresponding hexose-pentosides at each of the time points and broken green lines (▲) represent calculated amounts of corresponding hexose-pentosides cleaved at each of the time points. Time points at which there is a significant difference (the Student’s t-test, p<0.05) between the amount of volatiles emitted (Δ “volatile”(●)) and the the calculated amount of the corresponding glycosides cleaved (Δ “volatile” HP(▲)) are indicated with asterisks (*). Eugenol was not detected ( nd, panel C) at time points 3 and 6 minutes.

Figure 4. Dynamics of PhP-V glycosides in low PhP-V fruits upon fruit cell disruption. Ground and frozen powder of red fruits from 6 low PhP-V genotypes was incubated for 0, 3, 6, 12 and 24 minutes at 30°C (which included thawing). At each time point, levels of PhP-V glycosides: guaiacol dihexose-pentoside (Gua dHP) and malonyl dihexose-pentoside (Gua MdHP), methyl salicylate dihexose-pentoside (MeSA dHP) and malonyl dihexose pentoside (MeSA MdHP), dihexose-pentoside (Eug dHP) and malonyl dihexose-pentoside (Eug MdHP) were measured using LC-QTOF-MS, respectively. Abundances are represented as percentage of abundance of each of the compounds at time point 0 minutes. Gua dHP and Gua MdHP consist of 0.066 ± 0.014 ppm of bound guaiacol, MeSA dHP and MeSA MdHP – 0.098 ± 0.009 ppm of bound MeSA, Eug dHP and Eug MdHP – 0.53 ± 0.034 ppm of bound eugenol at starting time point 0. Average patterns (+/- SD) of 6 low PhP-V genotypes are presented. Multiple ANOVA test was performed to estimate the difference between time points of each of the compounds and corresponding p-values are presented on the charts.

Figure 5. Spiking of blended tomato fruit with high PhP-V glycosidic extracts
High (A) and low (B) PhP-V fruit matrix was spiked with a crude glycoside extract obtained from high PhP-V fruits and consisting of PhP-V diglycosides (hexose-pentosides, HP). Emission of volatiles was measured using GC-MS, reduction of PhP-V diglycosides was measured using LC-QTOF-MS and expressed as ppm volatiles emitted, as described in Materials and Methods. White bars: non-spiked control samples; Grey bars: additional amount of volatiles emitted or glycosides (HP) hydrolyzed in spiked samples.
Figure 6. Spiking of high PhP-V fruits blended with glycoside extract obtained from low PhP-V fruits, containing tri- and malonyl triglycosides of guaiacol, MeSA and eugenol.

A – relative amounts of PhP-V triglycosides in blended high PhP-V fruit spiked with a triglycoside extract, measured before and after incubation for 10 min at 30°C. LC-MS signal intensities of malonylated and non-malonylated triglycosides of each of the volatiles were expressed as % of the levels in the non-incubated sample. The following calculated amounts of bound PhP volatiles (as triglycosides and malonyl triglycosides) were spiked: 0.28 ± 0.05 ppm of guaiacol, 0.44 ± 0.04 of MeSA and 3.38 ± 0.23 of eugenol,

B – amounts of PhP volatiles emitted from non-spiked high PhP-V fruits blended and incubated for 10 min at 30°C (control, white bars) and from blended high PhP-V fruits spiked with a glycoside extract containing tri- and malonyl trihexose-pentosides of guaiacol, MeSA and eugenol (grey bars),

C – reduction of PhP-V diglycosides after 10 minutes incubation at 30°C of blended high PhP-V fruits (white bars) or in blended high PhP fruits spiked with a triglycoside extract (grey bars). Reduction of PhP-V diglycosides was measured using LC-QTOF-MS and expressed as ppm volatiles emitted, as described in Materials and Methods.

Figure 7. Analysis of the PhP volatiles and their respective glycosides during tomato fruit development in high and low PhP-V genotypes.

Frozen powder of 3 high and 3 low PhP-V genotypes was incubated for 10’ at 30°C and levels of volatiles released (after 10’ incubation) and of their corresponding glycosides (in intact fruit, prior to incubation) were measured by GC-MS and LC-MS, respectively. Results show average patterns of high and low PhP-V genotypes. A, B, C – PhP volatiles: guaiacol, methyl salicylate and eugenol, respectively. D, E, F – PhP-V diglycosides (hexose-pentosides) of guaiacol, methyl salicylate and eugenol, respectively. G, H, I – PhP-V triglycosides (dHP) and malonyl triglycosides (MdHP) of guaiacol, methyl salicylate and eugenol, respectively in low PhP lines (High PhP lines showed no / negligible levels of triglycosides; results not presented). Abundance of triglycosides and malonyl triglycosides is presented as a percentage of the maximum intensity of a compound’s parent ion detected by LC-MS. At red ripe stage both triglycosides and malonyl triglycosides represent 0.066 ± 0.014 ppm of bound guaiacol, 0.098 ± 0.009 ppm of bound MeSA and 0.50 ± 0.034 ppm of bound eugenol, respectively. MG, B, T and R – tomato fruit developmental stages: mature green, breaker, turning and red ripe, respectively.

Figure 8. Model of PhP-volatile emission in low and high PhP-V genotypes, through a ripening-dependent diversification of PhP volatile glycoconjugate patterns.

PhP-V hexose-pentosides (HP) are present at mature green (MG), breaker (B), turning (T) and red ripe (R) stages of high PhP-V genotypes (A) as well as at mature green stage of low PhP-V
genotypes (B). These diglycosides can be cleaved upon fruit cell disruption, leading to emission of the corresponding volatile aglycones. Emission of PhP volatiles is arrested in fruits of low PhP-V genotypes from breaker stage onwards, due to conversion of PhP-V hexose-pentosides into PhP-V dihexose-pentosides (dHP) and malonyl dihexose-pentosides (MdHP), which are resistant to the endogenous tomato glycosylhydrolitic activity induced upon fruit tissue disruption.
Table I. LC-QTOF-MS, -MS/MS analysis of non-volatile compounds correlating with PhP volatiles.

| MMSR cluster ID | Retention time, min | correlated PhP volatiles (R) | Mass spectra derived by MMSR | Ion selected for MS/MS | observed mass (o.m.) | calculated mass (c.m.) | o.m./c.m., ppm | Elemental formula | Putative identity |
|-----------------|---------------------|-----------------------------|-----------------------------|------------------------|---------------------|-----------------------|-----------------|----------------|-----------------|
| LC1.1           | 29.51               | Eugenol (R = 0.8)           | 503, 457, 293, 504, 505, 113 | 503                    | 503.1747            | 503.1770              | -4.6            | C_{22}H_{32}O_{13} | Eugenol-hexose-pentose, FA adduct |
|                 |                     |                             |                             | 457                    | 457.1709            | 457.1715              | -1.3            | C_{21}H_{30}O_{11} | Eugenol-hexose-pentose |
|                 |                     |                             |                             | 293                    | 293.0868            | 293.0878              | -3.4            | C_{11}H_{18}O_{9}    | hexosepentose-2H_{2}O |
|                 |                     |                             |                             | 457                    | 457.1709            | 457.1715              | -1.4            | C_{21}H_{30}O_{11} | Eugenol hexose-pentose |
|                 |                     |                             |                             | 293                    | 293.0808            | 293.0878              | 0.7             | C_{11}H_{18}O_{9} | hexose-pentose-2H_{2}O |
|                 |                     |                             |                             | 163                    | 163.0766            | 163.0765              | 0.9             | C_{10}H_{12}O_{2} | eugenol |
| LC1.2           | 19.25               | Methyl salicylate (R = 0.76) | 491, 293, 381               | 491                    | 491.1386            | 491.1406              | -4.1            | C_{20}H_{28}O_{14} | methyl salicylate hexose-pentose, FA adduct |
|                 |                     |                             |                             | 445                    | 445.1347            | 445.1361              | -1.0            | C_{19}H_{26}O_{12} | methyl salicylate hexose-pentose |
|                 |                     |                             |                             | 293                    | 293.0874            | 293.0878              | -1.4            | C_{11}H_{16}O_{9} | hexose-pentose-2H_{2}O |
|                 |                     |                             |                             | 151                    | 151.0405            | 151.0401              | 2.9             | C_{9}H_{10}O_{3} | methyl salicylate |
| LC1.3           | 16.17               | Guaiacol (R = 0.78)        | 463, 417, 293               | 463                    | 463.1455            | 463.1457              | -0.5            | C_{19}H_{28}O_{13} | guaiacol hexose-pentose, FA adduct |
|                 |                     |                             |                             | 417                    | 417.1410            | 417.1402              | 1.9             | C_{18}H_{26}O_{11} | guaiacol hexose-pentose |
|                 |                     |                             |                             | 293                    | 293.0884            | 293.0878              | 2.0             | C_{11}H_{18}O_{9} | hexose-pentose-2H_{2}O |
|                 |                     |                             |                             | 417                    | 417.1415            | 417.1402              | 3.1             | C_{10}H_{20}O_{11} | guaiacol hexose-pentose |
|                 |                     |                             |                             | 293                    | 293.0873            | 293.0878              | 3.1             | C_{10}H_{20}O_{11} | guaiacol hexose-pentose |
| LC2.1           | 13.58               | nd                         | 579                         | 579                    | 579.1925            | 579.1930              | -0.9            | C_{23}H_{38}O_{16} | Guaiacol dihexose-pentose |
|                 |                     |                             |                             | 447                    | 447.1506            | 447.1506              | -1.8            | C_{22}H_{36}O_{12} | Guaiacol dihexose |
|                 |                     |                             |                             | 285                    | 285.0972            | 285.0978              | -2.1            | C_{13}H_{12}O_{7} | Guaiacol hexose |
| LC2.2           | 17.16               | nd                         | 665, 457, 447, 411          | 665                    | 665.1942            | 665.1934              | 1.2             | C_{24}H_{38}O_{19} | guaiacol malonyl dihexose-pentose |
|                 |                     |                             |                             | 579                    | 579.1927            | 579.1930              | -0.5            | C_{23}H_{36}O_{16} | guaiacol dihexose-pentose |
|                 |                     |                             |                             | 123                    | 123.0445            | 123.0452              | -5.7            | C_{7}H_{6}O_{2} | guaiacol |

Table continues on the next page.
Column 1 represents the ID of an ion fragment cluster derived by MMSR clustering, representing putative semi-polar non-volatile compounds, detected by LC-MS. The retention time of these putative compounds is indicated in column 2. Column 3 shows the identity of the volatile compounds correlated with the putative non-volatile compounds and the corresponding Pearson product correlation coefficients (R). Mass spectra of the non-volatile compounds and ion fragments of these mass spectra selected for MS/MS analyses are in columns 4 and 5, respectively. Ion fragments observed in MS/MS analyses are listed in column 6 and the next four columns represent results of putative identification of the observed ion fragments: elemental formulas predicted for the observed ion fragments (column 9), calculated masses of the elemental formulas predicted (column 7) and their deviations from the observed masses in ppm (column 8) and, finally, putative identities of the observed masses (column 10).

|    | 1   | 2      | 3      | 4          | 5          | 6          | 7          | 8          | 9          | 10                              |
|----|-----|--------|--------|------------|------------|------------|------------|------------|-----------|----------------------------------|
| LC2.3 | 14.95 | nd    | 607    | 607.1874  | 607.1879  | -0.8       | C_{28}H_{38}O_{17} | methyl salicylate dihexose-pentose |
|     |     |        |        | 455.1401  | 455.1395  | 1.2        | C_{17}H_{28}O_{14} | dihexose-pentose               |
|     |     |        |        | 323.0980  | 323.0973  | 2.1        | C_{12}H_{20}O_{10} | dihexose                        |
|     |     |        |        | 293.0873  | 293.0878  | -1.7       | C_{11}H_{18}O_{9} | hexose-pentose                 |
| LC2.4 | 18.61 | nd    | 693, 659 | 693.1902  | 693.1883  | 2.7        | C_{28}H_{38}O_{20} | methyl salicylate malonyl dihexose-pentose |
|     |     |        |        | 607.1878  | 607.1879  | -0.2       | C_{23}H_{36}O_{17} | methyl salicylate dihexose-pentose |
|     |     |        |        | 151.0411  | 151.0401  | 6.8        | C_{9}H_{8}O_{3} | methyl salicylate               |
| LC2.5 | 27.25 | nd    | 705 (single) | 705.2238  | 705.2247  | -1.3       | C_{30}H_{42}O_{19} | eugenol malonyl dihexose-pentose |
|     |     |        |        | 619.2260  | 619.2243  | 2.7        | C_{27}H_{40}O_{16} | eugenol dihexose-pentose        |
|     |     |        |        | 163.0773  | 163.0765  | 5.2        | C_{19}H_{12}O_{2} | eugenol                        |
| LC2.6 | 24.85 | nd    | 619    | 619.2236  | 619.2243  | -1.3       | C_{23}H_{40}O_{16} | eugenol dihexose-pentose        |
|     |     |        |        | 323.0978  | 323.0973  | 1.5        | C_{18}H_{30}O_{10} | dihexose                       |
|     |     |        |        | 455.1401  | 455.1395  | 1.2        | C_{11}H_{28}O_{14} | dihexose-pentose               |
|     |     |        |        | 293.0874  | 293.0878  | -1.4       | C_{11}H_{18}O_{9} | hexose-pentose                 |
|     |     |        |        | 163.0761  | 163.0765  | -2.5       | C_{10}H_{12}O_{2} | eugenol                        |
