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A metabolomics approach to identify factors influencing glucosinolate thermal degradation rates in *Brassica* vegetables

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ABSTRACT

Thermal processing of *Brassica* vegetables can lead to substantial loss of potential health-promoting glucosinolates (GLs). The extent of thermal degradation of a specific GL varies in different vegetables, possibly due to differences in the composition of other metabolites within the plant matrices. An untargeted metabolomics approach followed by random forest regression was applied to identify metabolites associated to thermal GL degradation in a segregating *Brassica oleracea* population. Out of 413 metabolites, 15 were associated with the degradation of glucobrassicin, six with that of glucoraphanin and two with both GLs. Among these 23 metabolites three were identified as flavonols (one kaempferol- and two quercetin-derivatives) and two as other GLs (4-methoxyglucobrassicin, gluconasturtiin). Twenty quantitative trait loci (QTLs) for these metabolites, which were associated with glucoraphanin and glucobrassicin degradation, were identified on linkage groups C01, C07 and C09. Two flavonols mapped on linkage groups C07 and C09 and co-localise with the QTL for GL degradation determined previously.

1. Introduction

Brassica vegetables contain a specific group of phytochemicals, glucosinolates (GLs), which are almost exclusively found in this plant family. GLs co-exist with an endogenous enzyme, myrosinase (E.C. 3.2.1.147), in the plant tissue but physically separated to avoid hydrolysis until tissue damage. Epidemiological and mechanistic studies have shown health protective effects of GLs and their enzymatic hydrolysis products against several types of cancer, for example colon, colorectal, breast, bladder and prostate cancer (Traka & Mithen, 2009). However, as a result of food processing of *Brassica* vegetables the amount of GLs can be lowered substantially and hence there is growing interest to minimise losses during vegetable processing. Several mechanisms lead to losses of GLs during food processing: (a) enzymatic breakdown of GLs, (b) leaching of GLs and breakdown products into the cooking water and (c) thermal degradation (Dekker, Verkerk, & Jongen, 2000). The term thermal degradation refers to the degradation solely induced by heat. Losses of 78% of the total GLs caused by thermal degradation were estimated during canning of red cabbage (Oerlemans, Barrett, Suades, Verkerk, & Dekker, 2006). Thermal degradation in Brussels sprouts reduced one type of GL, the indolic glucobrassicin by 60% after heating for 15 min at 100 °C (Dekker, Hennig, & Verkerk, 2009). It has been shown that the thermal degradation is dependent on the chemical structure of the GL (Hanschen, Rohn, Mewis, Schreiner, & Kroh, 2012; Oerlemans et al., 2006), but also on the reaction environment, i.e. vegetable matrix, since the thermal degradation of chemically identical GLs differs in different vegetables (Dekker et al., 2009). In that paper, the term "matrix" refers to metabolites and other components present in the vegetables, since fully ground tissue was used to determine the thermal degradation, influences of the cell walls and tissue structure could be excluded (Dekker et al., 2009). The metabolite composition of plants is (partly) genetically determined (Keurentjes et al., 2006), hence we hypothesised previously that thermal degradation of GLs is (partly) genetically regulated. Genetic effects of thermal degradation have recently been investigated by Hennig, Verkerk, Dekker, and Bonnema (2013), who identified quantitative trail loci (QTLs) explaining part of the variation in thermal degradation. QTL mapping is the association of quantitative traits with molecular markers and is one possibility to identify genetic regions affecting traits (Collard, Jahufer, Brouwer, & Pang, 2005). In another study it

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has been shown that also environmental factors like the growing season influence thermal degradation (Hennig, Verkerk, Bonnema, & Dekker, 2012). From these results, the question arises which genes underlie the identified QTLs and which mechanism influences glucosinolate thermal degradation.

The aim of this paper is to test if certain metabolites are associated with glucosinolate thermal degradation during food processing. This information can help to identify the genes underlying the degradation QTL and to form new hypothesis about the degradation mechanism(s). Since there is yet no knowledge on which metabolites can influence GL thermal degradation, an untargeted metabolomics approach was applied as a promising tool to associate differences in metabolite composition to differential GL thermal degradation. Metabolomics is an important comparative tool to study global metabolite levels of e.g. plant materials treated with various conditions. Untargeted metabolomics approaches are used to measure as many metabolites as possible to obtain patterns or fingerprints of processed food samples (Capanoglu, Beekwilder, Boyacioglu, Hall, & De Vos, 2008; Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009).

GL thermal degradation rate constants were previously determined by heating plant material for several heating time points. In this paper, a preliminary study was performed in a subset of samples to investigate the effect of heating on the overall metabolite profile and to test if the degradation of metabolites has to be taken into account to associate metabolite profiles with GL thermal degradation rate constants. In the subsequent main study more genotypes were investigated without heating and the obtained metabolite profiles were used to test for association with GL thermal degradation. If certain metabolites influence the degradation rate of GLs, breeding for changing the levels of these metabolites in the plant can result in improved stability of GLs during *Brassica* food processing. The screening and selection for genotypes by analysing their concentration of certain metabolites is less laborious than testing the GL degradation during food processing in different genotypes. Hence the knowledge of metabolites influencing GL thermal degradation will facilitate breeding for vegetables with a high retention of GLs during food processing and will help to maximise the health promoting effects of GLs in *Brassica* vegetables at the stage of consumption.

2. Materials and methods

2.1. 1Plant material

A Doubled Haploid (DH) population, developed by Bohuon, Keith, Parkin, Sharpe, and Lydiate (1996), was used to study GL thermal degradation. This population was developed by crossing two DH parents, a rapid-cycling Chinese kale line, *B. oleracea* var. *alboglabra* (A12DHd), and a Calabrese broccoli line, *B. oleracea* var. *italica* (GDDH33), through microspore culture of the F_1. Seeds were sown into soil, plants were transplanted into 19 cm diameter pots after two weeks, randomized and grown for 6 weeks after transplanting in a greenhouse in Wageningen (The Netherlands). A total of 100 DH lines of the DH population were grown in five replicates in spring 2009 (end of March till end of May) under natural light and temperature conditions. Temperatures ranged from 5 °C to 16 °C during the night and from 13 °C to 30 °C during the day. Fertilizers were given two to three times per week (electric conductivity 2.1). A subset of ten DH lines of the DH population, selected based on their GL degradation rates in 2009, was grown again in spring 2011 (end of March till end of May) in a greenhouse in Wageningen. Temperatures in the latter growing season ranged from 17 °C to 22 °C (night/day); however, on sunny and warm days the temperature could rise to 30 °C during the afternoon. Artificial light was applied if the natural photoperiod was shorter than 16 h.

Eight weeks after sowing, leaves without the petioles were harvested in the morning and transported on ice to the laboratory for further sample preparation. Harvesting of the 100 DH lines in spring 2009 was performed on four consecutive mornings, but all five biological replicates of the same DH line were harvested at the same day. Harvesting of plants grown in spring 2011 was performed in one morning. All leaves from the five plants per line were mixed to prepare one homogenous sample. Analysis of Variance (ANOVA) was performed to test for an influence of the sampling on the thermal degradation rate constants (k_d) using the software IBM SPSS Statistics 19. No significant influence of the harvesting day on the k_d values was found for the identified GLs glucobrassicin, sinigrin, progoitrin, gluconapin and neoglucobrassicin. Marginal differences were detected for the GLs glucoiberin and glucoraphanin (Hennig et al., 2013).

In order to study the GL thermal degradation as a sole mechanism, without enzymatic degradation, myrosinase was inactivated by microwave treatment at high power for a short time (Oerlemans et al., 2006; Verkerk & Dekker, 2004). Leaves were cut into pieces of about 3 × 3 cm, of which 75 g was placed into a plastic beaker and held on ice until microwave treatment. In total, five plastic beakers, each containing 75 g of leaves, were placed at the same time in a microwave at 900 W for 6 min. After the microwave treatment, samples were immediately cooled on ice and weight loss was recorded. As a control, a mix of 75 g cut leaves was prepared and directly frozen without microwave treatment. All samples were subsequently freeze dried. Dried samples were weighed to record the water loss, ground into a fine power and stored at −20 °C until further treatment.

2.2. Sample treatment

A schematic overview of the sample treatment is illustrated in Fig. S1 (Supplementary Material).

2.2.1. Preliminary study

A preliminary study was performed to investigate the effect of heating on the metabolite composition of three DH lines of the DH population grown in 2009 and 2011 (AG6105, AG1017 and AG6026). The lines were selected based on previously determined thermal degradation rate constants (k_d) of glucoraphanin (4-methylsulfinylbutyl-GL, compound 1 in Fig. 1) and glucobrassicin (Indol-3-ylmethyl-GL, compound 2 in Fig. 1) which were the GLs present in all lines of the DH population (Hennig et al., 2013). Thermal degradation rate constants were calculated by modelling the measured GL concentrations over the heating time at 100 °C (0, 15, 30 and 60 min) using a first order kinetics as described by Hennig et al. (2012). The degradation rate constant (k_d) describes the steepness of the degradation curve. Plant material that was not microwaved was analysed without heating (fresh), whereas the microwaved material (with inactivated myrosinase) was used for the heating study. Dried plant powder was reconstituted with MilliQ water to obtain 0.5 g plant material having the same water content as before the microwave treatment and drying. Closed tubes containing this plant material were then placed in a heating block at 100 °C for 0, 15, 30 and 60 min. The average time for samples to reach boiling point (100 °C) was 4 min. After heating, samples were quickly cooled on ice and stored at -20 °C till metabolite profiling.

2.2.2. Main study

Microwaved plant material was used in a previous study to determine thermal degradation rate constants (Hennig et al., 2013). The microwaved plant material of a subset of the DH population (85 DH lines were selected based on genetic differences) was