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Research article

# Differences in the enzymatic hydrolysis of glucosinolates increase the defense metabolite diversity in 19 *Arabidopsis thaliana* accessions

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ABSTRACT

Plants of the order Brassicales produce glucosinolates (GS), a group of secondary metabolites that are part of an elaborate defense system. But it is not the GS itself rather its enzymatic hydrolysis products that cause the bioactive effects protecting the plants against pests and pathogens. Thus the enzymatic hydrolysis and a variety of additional influential factors determine the structural outcome of the GS degradation process. To evaluate the possible diversity of defense metabolites a range of 19 *Arabidopsis thaliana* accessions were selected showing divergence in their geographical origin, in their phenotype, and in their GS profile. These particular accessions accumulate several alkenyl GS, hydroxyalkyl GS, methylthioalkyl GS, and methylsulfinylalkyl GS in their rosette leaves whereas the indole GS contents are relatively invariant, as analyzed by UHPLC-DAD. After tissue disruption the enzymatic formation of GS hydrolysis products was examined and breakdown products were identified and quantified by GC-MS. Great differences in the amount and structure of volatile enzymatic degradation products could be observed in the different accessions, with strong variation in formation of epithionitriles, nitriles, and isothiocyanates. The occurrence of specific GS hydrolysis products was put in relation to relative gene expression profiles of myrosinases and specifier proteins as measured by RT-qPCR, and in relation to relative protein abundance of epithiospecifier protein. Dependent on the different GS profiles and reliant on degradation protein abundance and composition the ecotypes strongly varied in their ability to form isothiocyanates, nitriles and epithionitriles, thus increasing the plants' equipment of defense metabolites.

## 1. Introduction

Plants have evolved several mechanisms to defend themselves against pests and pathogens. The order Brassicales forms a group of plant secondary metabolites, the glucosinolates (GS) that are part of one of the best studied defense systems. GS comprise a β-D-thioglucose group, a side chain derived from amino acid precursors, and a sulfonated oxime moiety and can be classified into aliphatic, aromatic, and indole GS (Halkier and Gershenzon, 2006; Hanschen et al., 2014). To date more than 130 GS have been identified in plants of the order Brassicales (Clarke, 2010; Agerbirk and Olsen, 2012). However, not GS themselves, but their enzymatic degradation products account for the bioactive effects of these plant metabolites. Amongst the latter are toxic effects on herbivores and plant pathogens (Wittstock and Burow, 2010) but also health beneficial and cancer preventive effects on humans after consumption of *Brassica* vegetables such as broccoli (Jeffery and Araya, 2009; Dinkova-Kostova and Kostov, 2012; Veeranki et al., 2015). Thereby, the enzymatic hydrolysis process represents the key to bioactivity of these metabolites. Within the plant, GS occur spatially separated from the enzyme myrosinase, a β-D-thioglucosidase (EC 3.2.1.147), thus preventing their degradation in the intact plant (Kissen et al., 2009). However, GS encounter the myrosinase after tissue disruption, which hydrolyses the β-D-thioglucosidic bond to release β-D-glucose and an instable aglucone, the thiohydroximate-*O*-sulfate. The latter spontaneously forms the isothiocyanate (ITC) by a Lossen rearrangement or decomposes to the nitrile (Bones and Rossiter, 2006). Formation of ITCs is favored at typical plant pH values (pH 5–6), while nitriles are preferentially formed at low pH values (< pH 4) or at increased $Fe^{2+}$-levels (Gil and Macleod, 1980; Uda et al., 1986). However, many Brassicaceae plants contain additional proteins that modify the breakdown of the aglucone to yield nitriles, epithionitriles (EPTs) or thiocyanates instead of ITCs (Kuchernig et al., 2012). Tookey (1973) isolated a protein - the epithiospecifier protein (ESP) from *Crambe abyssinica* which modifies the breakdown of unsaturated aliphatic GS to

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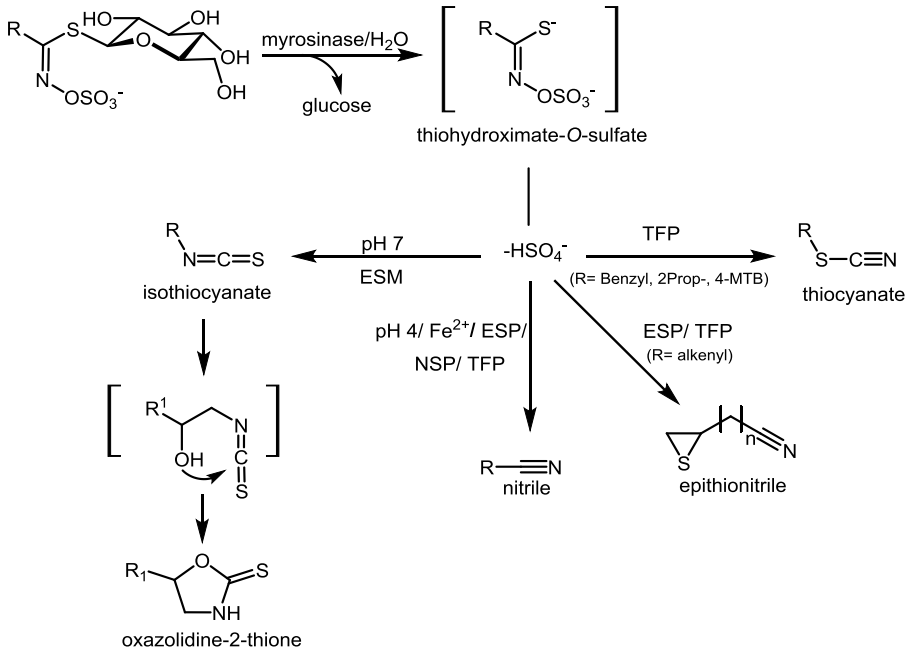


**Fig. 1. Exemplary enzymatic hydrolysis of glucosinolates after tissue disruption in *A. thaliana*.** 4-MTB, 4-methylsulfanyl-GS; 2Prop, 2-propenyl GS; ESM, epithiospecifier modifier; ESP, epithiospecifier protein; NSP, nitrile specifier protein; R, variable side chain; $R^1$, alkenyl side chain; TFP, thiocyanate-forming protein.

yield EPT via an intramolecular mechanism (Brocker and Benn, 1983). Additionally, if no terminal double bond is present, GS are favorably converted to nitriles. ESP protein occurs in many Brassicaceae plants, including *Brassica oleracea* such as broccoli, thereby decreasing putative health promoting effects derived from the ITC (Matusheski et al., 2006). ESP protein activity is dependent on $Fe^{2+}$ ions (Zabala et al., 2005; Williams et al., 2010) but also $Fe^{3+}$ was shown to catalyze EPT formation as well in model assays (Burow et al., 2006). The formation of GS-derived degradation products in the model plant *A. thaliana* is presented in Fig. 1.

One of the best studied plants of the Brassicaceae family is *A. thaliana*. The genetic variation at the *ESP* locus in Arabidopsis has been linked with varying herbivore resistance (Lambrix et al., 2001). Moreover, the epithiospecifier modifier (ESM) was shown to repress nitrile formation and favor ITC production thereby deterring herbivory (Zhang et al., 2006). Further, as L-(+)-ascorbic acid can increase myrosinase activity, higher myrosinase activity can favor the ITC formation as well (Burow et al., 2006). Contrarily, in Arabidopsis also five nitrile specifier proteins (NSPs) have been detected that promote the formation of simple nitriles (Wentzell and Kliebenstein, 2008; Burow et al., 2009). These NSPs were shown to be responsible for nitrile formation in root, seed, and seedling homogenates of *A. thaliana* (Wittstock et al., 2016). Their activity can increase with $Fe^{2+}$, while NSP1 activity seems to be not strictly dependent on $Fe^{2+}$ (Kong et al., 2012).

In addition, the thiocyanate forming-protein, which has similarities to ESP in amino acid sequence and $Fe^{2+}$ dependency, catalyzes simple nitrile and EPT formation from the aglucon of aliphatic GS and forms organic thiocyanates from benzyl-, allyl- and 4-methylsulfanyl-GS in *Lepidium sativum* (Hasapis and Macleod, 1982; Burow et al., 2007a). With the help of mutational analysis and quantum-mechanical calculations, a mechanism for thiocyanate formation has been proposed (Brandt et al., 2014). Recently, also the crystal structure of AtESP was solved (Zhang et al., 2016).

Thus, enzymatic activity and formation of hydrolysis products depends on a variety of factors that influences the structural outcome of GS degradation. We are at the beginning to understand how all these proteins interact and determine the hydrolysis product formed. In an approach to further elucidate the degradation diversity of GS in *A. thaliana*, 19 accessions have been selected that differ in their origin, phenotype, genetic variation, as well as in their GS profile (Kover et al., 2009; Witzel et al., 2013). These accessions are part of the multiple reference genomes and transcriptomes for *A. thaliana* (Gan et al., 2011) and available genome information can be easily implemented. The formation of GS hydrolysis products was correlated with the gene expression profile of myrosinases and specifier proteins in order to study their interaction in the enzymatic degradation pathway.

## 2. Methods

### 2.1. Chemicals

2-propenyl isothiocyanate (2Prop-ITC, ≥ 99%), benzonitrile (≥99.9%), 3-butenenitrile (2Prop-CN, ≥ 98%), 4-pentenenitrile (3But-CN, ≥ 97%), 3-phenylpropanenitrile (2PE-CN, ≥ 99%), 2-propenyl glucosinolate hydrate (2Prop GS, ≥ 99%), and 3-(methylsulfanyl) propyl ITC (3MTP-ITC, ≥ 98%), ethylenediaminetetraacetic acid, serum albumin, 5-bromo-4-chloro-3-indolyl (BCIP) and nitroblue tetrazolium chloride (NBT) were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany; 2-phenylethyl isothiocyanate (2PE-ITC; ≥ 99%) was purchased from SAFC Supply Solutions, St. Louis, Missouri, USA; Benzyl ITC (≥97%) and phenylacetonitrile (Benzyl-CN, ≥ 98%) from Lancaster Synthesis Ltd., Morecambe, UK; 3-indoleacetonitrile (IAN, ≥ 98%) from Acros Organics (Fischer Scientific GmbH, Schwerte, Germany); $HNO_3$ (65%, Suprapur®), $H_2O_2$ (30%, Suprapur®), and metaphosphoric acid was purchased from Merck KGaA, Darmstadt, Germany; 3-butenyl ITC (3But-ITC, ≥ 95%) and 4-pentenyl ITC (4Pen-ITC, ≥ 95%) were purchased from TCI Deutschland GmbH, Eschborn, Germany; 3-hydroxypropionitrile was purchased from Thermo Fischer Scientific, Erembodegem, Belgium; 3-(methylsulfinyl)propyl ITC (3MSOP-ITC, ≥ 97%) and 4-(methylsulfanyl)butyl ITC (4MTB-ITC, ≥ 98%) were purchased from Santa Cruz Biotechnology, Heidelberg, Germany; 4-(methylsulfinyl)butyl ITC (4MSOB-ITC, ≥ 98%) and 5-(methylsulfinyl)butyl ITC (5MSOP-ITC) were purchased from Enzo Life Sciences GmbH, Lörrach, Germany; 3,4-epithiobutanenitrile (CETP, ≥ 95%) was purchased from Taros Chemicals GmbH Co. KG, Dortmund, Germany; methylene chloride (GC Ultra Grade) from Carl Roth GmbH, Karlsruhe, Germany; acetonitrile (Ultra Gradient HPLC grade) was purchased from J.T. Baker, Deventer, Nederland; $NaSO_4$ (≥99%) and dithiothreitol were purchased from VWR International GmbH, Darmstadt, Germany.