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# Influence of Seasonal Variation and Methyl Jasmonate Mediated Induction of Glucosinolate Biosynthesis on Quinone Reductase Activity in Broccoli Florets

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Supporting Information

**ABSTRACT:** Methyl jasmonate spray treatments (250 $\mu$M) were utilized to alter glucosinolate composition in the florets of the commercial broccoli $F_1$ hybrids 'Pirate', 'Expo', 'Green Magic', 'Imperial', and 'Gypsy' grown in replicated field plantings in 2009 and 2010. MeJA treatment significantly increased glucoraphanin (11%), gluconasturtiin (59%), and neoglucobrassicin (248%) concentrations and their hydrolysis products including sulforaphane (152%), phenethyl isothiocyanate (318%), *N*-methoxyindole-3-carbinol (313%), and neoascorbigen (232%) extracted from florets of these genotypes over two seasons. Increased quinone reductase (QR) activity was significantly correlated with increased levels of sulforaphane, *N*-methoxyindole-3-carbinol, and neoascorbigen. Partitioning experiment-wide trait variances indicated that the variability in concentrations of sulforaphane (29%), neoascorbigen (48%), and QR activity (72%) was influenced by year-associated weather variables, whereas variation in neoglucobrassicin (63%) and *N*-methoxyindole-3-carbinol (46%) concentrations was primarily attributed to methyl jasmonate treatment. These results suggest that methyl jasmonate treatment can enhance QR inducing activity by increased hydrolysis of glucoraphanin into sulforaphane and the hydrolysis products of neoglucobrassicin.

**KEYWORDS:** *Brassica oleracea var. italica, anticancer activity, glucosinolate, sulforaphane, N-methoxyindole-3-carbinol, neoascorbigen, environmental stress*

## INTRODUCTION

Broccoli (*Brassica oleracea* ssp. *italica*) is one of the most frequently consumed vegetables in the United States and in other countries. Broccoli is well-known for its health-promoting bioactivity, with previous research reporting that regular consumption of this vegetable is associated with the prevention of prostrate, colon, breast, lung, and skin cancer.[1−6] Moreover, epidemiological studies have reported that dietary consumption of *Brassica* vegetables is inversely correlated with cancer risk, and this association is stronger than those between cancer and fruit and vegetable consumption in general.[7]

Diet is one of the most important factors in carcinogenesis accounting for approximately 47% of the variation in cancer risk among the nonsmoking public.[8] Certain phytochemicals have anticarcinogenic activity and induce phase II detoxifying enzymes in mammals including glutathione *S*-transferase (GST) and quinone reductase (QR) that can enhance detoxification and elimination of carcinogens from the body.[9] Upregulation of QR activity has been used as a biomarker for cancer prevention because this enzyme is a catalyst for the conversion of quinones into stable and nontoxic hydroquinones, reducing oxidative cycling.[10] Moreover, QR activity elevation in in vitro and in vivo model systems has been shown to correlate with induction of other protective phase II enzymes such as GST and provides a reasonable biomarker for the potential chemoprotective effect of test agents against cancer initiation and proliferation.[11]

Glucosinolates (GS) are secondary metabolites existing in almost all plants of the order *Brassicales*. Although intact GS do not have strong bioactivity, products of GS generated by hydrolysis by the endogenous enzyme myrosinase in broccoli have been shown to enhance QR and other health-promoting activities. Among the GS products, sulforaphane (**1b**, Figure 1), an isothiocyanate generated from the hydrolysis of glucoraphanin (**1a**), is a potent QR inducer and is considered to be an active agent in the prevention of certain cancers.[4] Phenethyl isothiocyanate (**3b**), an isothiocyanate derived from the hydrolysis of the aromatic GS (**3a**), also induces synthesis of the QR enzyme[12] and has been shown to protect against colon cancer in rats.[13] *N*-Methoxyindole-3-carbinol (**5b1**), the hydrolysis product of the indolyl GS, neoglucobrassicin (**5a**), has been reported to induce cell cycle arrest in human colon cancer cell lines resulting in reduced initiation and tumor growth.[14]

The GS are also associated with insect defense in *Brassica* species. Jasmonic acid (JA), an endogenous plant signal transduction compound whose biosynthesis is upregulated when *Brassica* plant species are attacked by herbivores, causes enhanced indolyl GS biosynthesis.[15] It has been reported that the indolyl GS whose biosynthesis is upregulated by methyl jasmonate treatment in broccoli is predominately **5a** and, to a lesser extent, **3a**.[16] **3b** derived from gluconasturtiin (**3a**) and **5b1**

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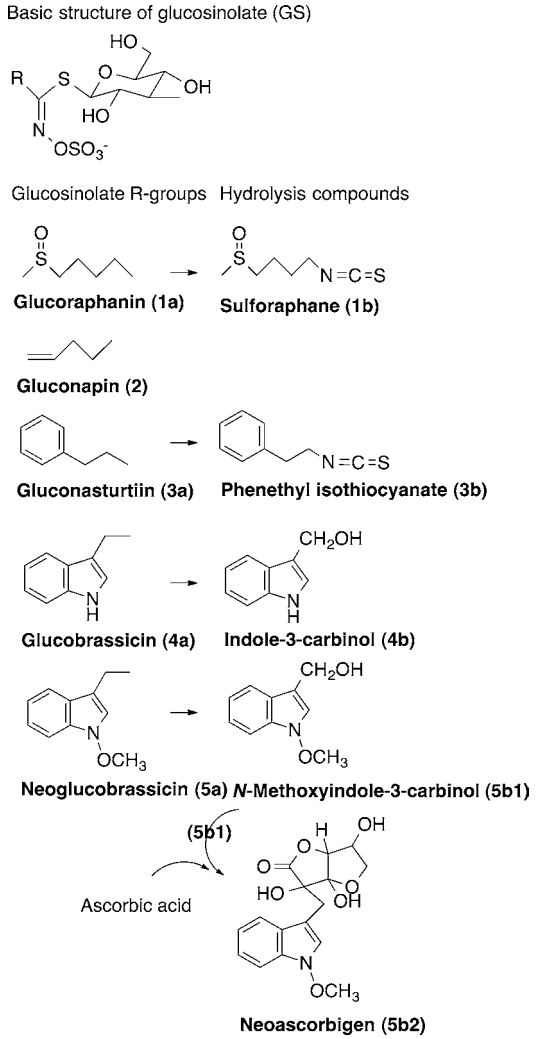


**Figure 1.** Structure of measured glucosinolates (GS) and their hydrolysis products in broccoli.

derived from **5a** have previously been reported as QR inducers and anticancer agents.[14,17,18] In addition to **5b1**, neoascorbigen (**5b2**) can be generated from **5a** hydrolysis by condensation with ascorbic acid (Figure 1).[19] However, there is little information about the health effects of **5b2** and the variation of **5b2** concentrations associated with biotic and abiotic stresses. While there are many previous reports of methyl jasmonate mediated increases in GS concentrations, few of these studies investigate how these treatments influence abundance and activities of GS hydrolysis products which are directly associated with anticancer activity.

The objective of this research was to investigate which of the GS and their hydrolysis products are primarily associated with the enhanced QR induction mediated by methyl jasmonate treatments. Variance in QR activity was partitioned by ANOVA into sources of variation associated with methyl jasmonate treatment, genotype, and environment (year) main factors and their interactions. Correlation analysis was conducted to test if QR inductive activity shows meaningful correlations with GS hydrolysis products and weather-related environmental conditions over different production seasons. This information is useful for the identification of superior broccoli germplasm and for selection strategies in *Brassica* breeding programs designed to develop cultivars with enhanced health-promoting properties.

## METHODS AND MATERIALS

**Broccoli Cultivation.** The five $F_1$ hybrid broccoli cultivars used in this experiment were 'Pirate' (Asgrow Seed Co., Galena, MD), 'Expo', 'Imperial', 'Gypsy', and 'Green Magic' (Sakata Seed Co., Morgan Hill, CA). Broccoli cultivation and experiment design have already been published in a previous study.[20] Weather data used in this study during the 2009 and 2010 growing seasons is available online.[21] Since accumulated solar radiation and precipitation [(PPT)/number of days from transplant to harvest (DTH)] varied between years and the number of growing degree days (GDD) [(minT + maxT)/2−7.2 °C][22] varied for each genotype, these values were calculated for each year and genotype separately (Table 1).

**Table 1. Days from Transplant to Harvest (DTH) and Accumulated Growing Degree Days (GDD), Solar Radiation, and Precipitation Experienced by Each of the Five Broccoli Genotypes in 2009 and 2010**

| year | cultivar | treatment | DTH | GDD (°C) | solar radiation (mJ/m$^2$) | precipitation (mm) |
|---|---|---|---|---|---|---|
| 2009 | Expo | control | 81 ± 3 | 1134 | 1705 | 296 |
| | | methyl jasmonate | 82 ± 3 | 1147 | 1729 | 296 |
| 2009 | Green Magic | control | 58 ± 7 | 847 | 1257 | 223 |
| | | methyl jasmonate | 57 ± 7 | 835 | 1239 | 222 |
| 2009 | Gypsy | control | 62 ± 5 | 894 | 1342 | 223 |
| | | methyl jasmonate | 61 ± 2 | 881 | 1318 | 223 |
| 2009 | Imperial | control | 60 ± 3 | 869 | 1293 | 223 |
| | | methyl jasmonate | 60 ± 2 | 869 | 1293 | 223 |
| 2009 | Pirate | control | 77 ± 5 | 1079 | 1622 | 296 |
| | | methyl jasmonate | 78 ± 5 | 1093 | 1643 | 296 |
| 2010 | Expo | control | 88 ± 2 | 1513 | 2758 | 314 |
| | | methyl jasmonate | 93 ± 3 | 1576 | 2868 | 318 |
| 2010 | Green Magic | control | 67 ± 6 | 1185 | 2165 | 245 |
| | | methyl jasmonate | 67 ± 6 | 1185 | 2165 | 245 |
| 2010 | Gypsy | control | 68 ± 3 | 1201 | 2193 | 245 |
| | | methyl jasmonate | 69 ± 2 | 1217 | 2223 | 245 |
| 2010 | Imperial | control | 65 ± 2 | 1094 | 2100 | 245 |
| | | methyl jasmonate | 66 ± 4 | 1169 | 2136 | 245 |
| 2010 | Pirate | control | 92 ± 6 | 1563 | 2825 | 314 |
| | | methyl jasmonate | 91 ± 4 | 1552 | 2845 | 314 |


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**Broccoli Treatment with Methyl Jasmonate and Sample Preparation.** An aqueous solution of 250 $\mu$M methyl jasmonate (Sigma-Aldrich, St. Louis, MO) and 0.1% Triton X-100 (Sigma-Aldrich) in distilled water was sprayed on all aerial plant tissues to the point of runoff (approximately 300 mL) four days prior to harvest at commercial maturity. For the control group, 0.1% Triton X-100 was sprayed on plant as described above. We have already tested different application dates, concentrations, and surfactants to maximize methyl jasmonate mediated biosynthesis of glucosinolates.[23] Five broccoli heads were harvested from treatments and controls of each genotype for each replicate. Broccoli heads were frozen in liquid nitrogen and stored at −20 °C prior to freeze-drying. Freeze-dried head tissues were ground into a fine powder using a coffee grinder and stored at −20 °C prior to chemical and bioactivity analyses.

**Isolation of 5a and Generation of Hydrolysis Products.** In order to measure concentrations of hydrolysis products of **5a** from different cultivars with or without methyl jasmonate treatment and their QR inducing activity, **5a** was isolated and purified from broccoli following a previously described protocol.[24] Two grams of methyl jasmonate-treated 'Green Magic' broccoli powder was extracted with 10 mL of 70% methanol in a 50 mL Falcon conical centrifuge tube (BD Biosciences, San Jose, CA) for 10 min. After cooling, the supernatant obtained following lead/barium acetate precipitation was loaded onto a 20 cm × 2 cm ion exchange column containing Sephadex A-25 (Sigma-Aldrich) and indolyl GS eluted from the column with 0.02 M pyridine acetate (20 mL) and 0.25 M pyridine acetate (20 mL). The eluent fractions from ion exchange chromatography containing **5a** were dried using a SpeedVac AES2010 concentrator (Thermo Savant, Waltham, MA) and quantitated on a Agilent 1100 HPLC system (Agilent, Santa Clara, CA), equipped with a G1311A binary pump, a G1322A vacuum degasser, a G1316A thermostatic column compartment, a G1315B diode array detector, and an HP 1100 series G1313A autosampler. Extracts were separated on a Supercosil LC-18 column (250 × 4 mm, particle size 5 $\mu$m, Supelco Inc., Bellefonte, PA) with a 7.5 mm × 4.6 mm i.d., 5 $\mu$m Adsorbosphere C18 guard column (Grace, Deerfield, IL). Mobile phase A was water and B methanol. Mobile phase B was 0% at injection and held 4 min, increasing to 15% by 10 min, 35% at 20 min, and 80% at 21 min, then held 4 min, then decreased to 0% by 30 min. Flow rates were kept at 1 mL/min. The detector wavelength was set at 227 nm. The concentration of **5a** was determined using benzylglucosinolate as an internal desulfoglucosinolate standard. Purity was over 98%.

Hydrolysis products of **5a** including **5b1** and **5b2** were generated by incubation of 20 g of freeze-dried methyl jasmonate-treated 'Green Magic' broccoli in 100 mL of pH 8 distilled water without ascorbic acid or pH 5.6 distilled water with 1 mM ascorbic acid, respectively. After 4 h incubation at room temperature, 100 mL of methylene chloride was added and shaken. The emulsion was transferred to a 50 mL tube and centrifuged at 5000*g* for 10 min. The organic layer was carefully collected, and traces of water were removed using sodium sulfate. After filtration with Whatman No. 1 paper filter (GE Healthcare Life Sciences, Piscataway, NJ), the methylene chloride extract was dried with nitrogen gas. The dried samples from each hydrolysis pH treatment were dissolved in 1.5 mL of 50% acetonitrile and filtered with a 0.45 $\mu$m polytetrafluoroethylene syringe filter (13 mm) (Fisher Scientific, Waltham, MA). The solution was subjected to separation and fractionation using the same methods described above for the isolation of **5a**.

**Tentative Identification of Purified Compounds.** Identification of desulfated GS was achieved using a Q-TOF Ultima electrospray ionization (ESI) mass spectrometer (MS) and MS/MS (Waters, Milford, MA). The ESI MS was operated in positive ion mode with source conditions set at capillary voltage 3 kV; cone voltage 35 V; source temperature 120 °C; desolvation temperature 375 °C; and collision energy 12 eV. Identification of the hydrolysis products of **5a** was achieved using electron impact (EI) direct inlet MS using a Micromass 70-VSE (Waters) double-focusing magnetic sector mass spectrometer in positive ion mode at 70 eV and a source temperature of 30 °C. The instrument was scanned from $m/z$ 50 to 400. High-resolution mass spectrometry was performed on the same instrument as above.

**Determination of Sample GS Concentrations.** Extraction and quantification of GS using high-performance liquid chromatography was performed using a previous protocol.[25] Benzylglucosinolate was used as an internal standard, and UV response factors for different types of GS were used as determined by previous study[26] to calculate concentrations. The identification of desulfo-GS profiles were validated by LC−tandem MS using a Waters Q-TOF Ultima spectrometer coupled to a Waters 1525 HPLC system and full scan LC−MS using a Finnigan LCQ Deca XP, respectively.[27,28]

**Analysis of Glucosinolate Hydrolysis Products.** The extraction and analysis of isothiocyanates and other hydrolysis products was carried out according to previously published methods, with some modifications.[29] In order to determine the appropriate time for maximum GS hydrolysis by endogenous sample myrosinase, concentrations of hydrolysis products were quantitated in a preliminary experiment using extracts from the 'Green Magic' at various time points. Based on the preliminary results using 'Green Magic' cultivar, hydrolysis product concentrations of all samples were quantitated at 2, 4, 16, 24, and 28 h using aliquots by HPLC. 75 mg of broccoli powder was suspended in 1.5 mL of water in the absence of light for 4 h (time for the maximum concentration of indolyl GS hydrolysis products) at room temperature in a sealed 2 mL microcentrifuge tube to facilitate GS hydrolysis by endogenous myrosinase. Slurries were then centrifuged at 12000*g* for 5 min, and supernatants were decanted into a 2 mL microcentrifuge tube. 20 $\mu$L of butyl isothiocyanate (0.5 mg/mL) and 4-methoxyindole (1 mg/mL) were added as the internal standards for isothiocyanates and hydrolysis products of indolyl GS to quantitate indole-3-carbinol (**4b**), **5b1**, and **5b2** with 0.5 mL of methylene chloride. Tubes were shaken vigorously before being centrifuged for 2 min at 9600*g*. The methylene chloride layer (200 $\mu$L) was transferred to a 350 $\mu$L flat bottom insert (Fisher Scientific, Pittsburgh, PA) in a 2 mL HPLC autosampler vial (Agilent, Santa Clara, CA) for mixing with 100 $\mu$L of a reagent containing 20 mM triethylamine and 200 mM $\beta$-mercaptoethanol in methylene chloride. For **1b** and **3b**, unlike other hydrolysis products of GS, 0.5 mL of fresh broccoli extracts was kept mixed with 0.5 mL of derivatization reagent using an orbital shaker at 220 rpm for 24 h. The mixture was incubated at 30 °C for 60 min under constant stirring and then dried under a stream of nitrogen. The residue containing isothiocyanate derivatives (isothiocyanate-mercaptoethanol derivatives), other hydrolysis compounds were dissolved in 200 $\mu$L of acetonitrile/water (1:1) (v/v), and 10 $\mu$L of this solution was injected onto an Agilent 1100 HPLC system. Extracts were separated on an Eclipse XDB-C18 column (150 × 4 mm i.d., 5 $\mu$m, Agilent, Santa Clara, CA) with a 7.5 mm × 4.6 mm i.d., 5 $\mu$m Adsorbosphere C18 guard column. Mobile phase A was water, and B was methanol. Mobile phase B was 0% at injection, increasing to 10% by 10 min, 100% at 35 min, and held 5 min, then decreased to 0% by 50 min. Flow rates were kept at 0.8 mL/min. The detector was set at wavelength 227 and 271 nm. Response factors for monomeric indolyl derivatives were used from a previous report.[19] Due to a lack of standards for **5b1** and **5b2** the standard curve of **4b** was applied for quantification of both **5b1** and **5b2**. The quantities were expressed as **4b** molar equivalent concentrations.

**Quinone Reductase (QR) Activity.** For the QR assay, broccoli extracts were collected using the same protocol for GS hydrolysis products described above with sampling at 30 min, 4 h (peak concentrations for **5a** hydrolysis products), and 24 h (peak concentrations for **1b**) of incubation. The QR induction activities of different samples were determined by means of the protocol described by Prochaska and Santamaria.[30]

**QR Activity Measurement of Hydrolysis Products of 5a.** QR activity of hydrolysis products of purified **5a** were measured after hydrolysis with 0.5 U/mL (final concentration) commercial *Sinapis alba* myrosinase (Sigma-Aldrich) and **5a** (700 $\mu$M) for 40 min in pH 7.2 of phosphate buffered saline (PBS) without ascorbic acid or pH 5.6 of PBS with 1 mM ascorbic acid to produce different forms of hydrolysis products, respectively, and observed until completion of the hydrolysis by HPLC as described above (method for the hydrolysis

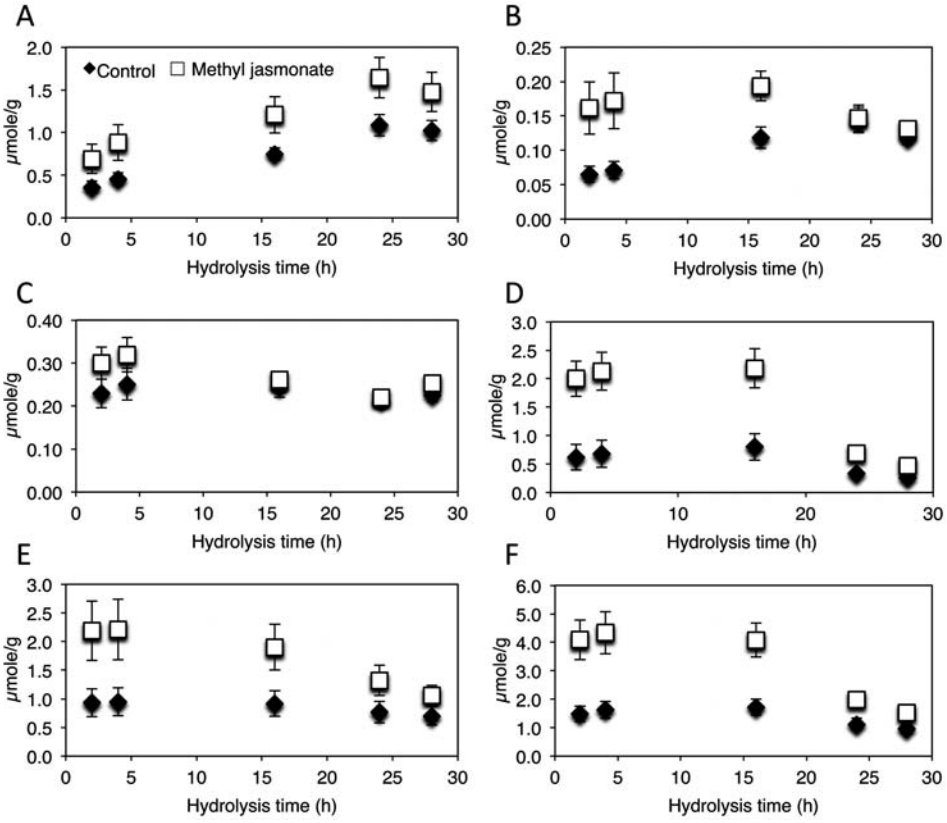


**Figure 2.** Time course to determine maximum concentration for each of the hydrolysis products of GS. Mean ± SEM ($n$ = 10). A: **1b**. B: **3b**. C: **4b**. D: **5b1**. E: **5b2**. F: **5b1** + **5b2**.

products analysis). The reaction products were left on ice prior to QR assays. Different concentrations of hydrolysis products were added to the media containing hepa1c1c7 cells to determine the concentration required to double QR induction values.

**Statistical Analysis.** Analysis of variance (ANOVA) and partitioning of variance components for phytochemicals and QR activity using total sums of squares were conducted using JMP 10 (SAS institute Inc., Cary, NC). Year, treatments, and genotype effects were considered as fixed factors. Block was considered as random. Correlation analysis and Student's $t$ tests were also conducted using the JMP 10 software. All sample analyses were conducted in triplicate. The results are presented as means ± SD.

## RESULTS AND DISCUSSION

**Tentative Identification of 5a and Its Hydrolysis Products.** In order to quantitate hydrolysis products, **5a** was purified from methyl jasmonate treated 'Green Magic' floret tissue. The purified **5a** and its hydrolysis products, **5b1** and **5b2**, were confirmed by comparison with literature data.[17,19,31,32]

**Time Course of GS Hydrolysis and QR Inducing Activity.** Some GS hydrolysis products are relatively unstable in aqueous extracts including **5b1** and **4b**. To determine the optimal time for maximum **5a** hydrolysis, hydrolysis product concentrations were sampled and quantitated at a range of time points. The maximum concentrations of each hydrolysis product were found to be 4 h for **5b2** and **4b**, 16 h for **5b1** and **3b**, and, 24 h for **1b** (Figure 2). Compared to the amount of precursor GS, accumulated concentrations of **3b**, the hydrolysis product of **3a**, were relatively low (Figure 2C). It is reported that **3b** is relatively volatile and has very low solubility in water (0.051 mg/mL) compared to **1b** (8.0 mg/mL).[33] Previous studies could not detect **3b** in hydrolyzed watercress extracts.[18,34] To eliminate volatility issues, **1b** and **3b** in Table 2 were measured after shaking with isothiocyanate derivatization reagent for 24 h.

The different peak times for hydrolysis accumulation of **1b** and the hydrolysis products of indolyl GS may be due to variation in isoforms of myrosinase in broccoli. James and Rossiter reported[35] that there were two isoforms of myrosinase in *Brassica napus* and their hydrolysis efficiency varied between aliphatic and indolyl GS. An atypical myrosinase, PEN2, has been identified which cleaves indolyl GS in planta preferentially as a mechanism of phytochemical defense against fungal pathogens.[36]

QR activity was tested using all samples (five genotypes with or without methyl jasmonate treatment over two years) with sample aliquots from different time points of hydrolysis at 30 min, 4 h, and 24 h. The 4 h hydrolysis aliquots displayed 97% of QR activity observed after 24 h of hydrolysis. Previous research reported that full induction of the QR enzyme required at least 6 h exposure of elicitor.[37] **5b1** and **5b2** were observed to rapidly degrade after 16 h. Considering the relatively higher concentrations of **5b2** and **5b1** compared to other hydrolysis products and their instability we suggest that QR inducing activity be assayed between 4 and 16 h after sample hydrolysis.

**Effect of Methyl Jasmonate Treatment on GS Concentrations in Broccoli Florets.** Over both seasons 250 μM methyl jasmonate treatments were observed to significantly increase **1a** (11%), **3a** (60%), and **5a** (248%) floret tissue concentrations across cultivars (Table 2). **2** was significantly decreased (53%) by methyl jasmonate treatment across two seasons in the five broccoli cultivars (Table 2). Since **1a** is upstream in the aliphatic GS biosynthesis pathway, decreased **2** concentration may partly be due to the increase of **1a** or from a


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**Table 2. Concentrations of Glucosinolates (μmol per g of DW), Their Hydrolysis Products, and QR Inducing Activity (Specific Activity Ratio of Broccoli Extract Treated Cells to Non-Treated Cells) in Untreated (Control) and Methyl Jasmonate Treated Broccoli**

| source of variation | treatment | 1a | 1b | 2 | 3a | 3b | 4a | 4b | 5a | 5b1[a] | 5b2[a] | 5b1 + 5b2[a] | total GS[b] | QR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| treatment | | | | | | | | | | | | | | |
| | control | 4.00 ± 1.86 | 1.08 ± 0.31 | 1.90 ± 1.47 | 1.68 ± 0.71 | 0.40 ± 0.22 | 2.67 ± 0.77 | 0.25 ± 0.12 | 2.88 ± 1.43 | 0.68 ± 0.34 | 0.95 ± 0.75 | 1.63 ± 0.95 | 13.6 ± 3.85 | 3.20 ± 0.64 |
| | methyl jasmonate | 4.42 ± 2.01*[c] | 1.64 ± 0.60* | 1.24 ± 0.88* | 2.68 ± 1.12* | 1.27 ± 0.55* | 2.64 ± 1.01 | 0.32 ± 0.13* | 10.0 ± 3.71* | 2.13 ± 1.08* | 2.21 ± 1.65* | 4.34 ± 2.36* | 21.5 ± 6.43* | 3.53 ± 0.82* |
| year | | | | | | | | | | | | | | |
| 2009 | control | 3.67 ± 0.64 | 1.08 ± 0.28 | 0.80 ± 0.39* | 1.37 ± 0.55 | 0.41 ± 0.23 | 2.90 ± 0.66 | 0.26 ± 0.14 | 1.98 ± 1.02 | 0.65 ± 0.31 | 0.31 ± 0.13 | 0.96 ± 0.41 | 11.2 ± 2.2 | 2.65 ± 0.40 |
| | methyl jasmonate | 4.01 ± 1.19 | 1.22 ± 0.47* | 0.57 ± 0.47 | 2.41 ± 1.25* | 1.42 ± 0.57* | 3.28 ± 1.00* | 0.34 ± 0.17* | 10.8 ± 3.98* | 1.85 ± 1.21* | 0.90 ± 0.48* | 2.74 ± 0.99* | 21.5 ± 6.7* | 2.83 ± 0.45* |
| 2010 | control | 4.32 ± 2.59 | 1.15 ± 0.34 | 3.00 ± 1.31* | 1.99 ± 0.73 | 0.38 ± 0.22 | 2.43 ± 0.83 | 0.24 ± 0.09 | 3.79 ± 1.21 | 0.71 ± 0.40 | 1.59 ± 0.53 | 2.29 ± 1.30 | 16.0 ± 3.7 | 3.75 ± 0.31 |
| | methyl jasmonate | 4.83 ± 2.57 | 1.88 ± 0.66* | 1.91 ± 0.64 | 2.96 ± 0.93* | 1.11 ± 0.50* | 1.99 ± 0.48 | 0.30 ± 0.08* | 9.30 ± 3.38* | 2.41 ± 0.87* | 3.53 ± 1.30* | 5.95 ± 1.90* | 21.4 ± 6.4* | 4.23 ± 0.25* |
| genotypes 2009 | | | | | | | | | | | | | | |
| Pirate | control | 2.99 ± 0.32 | 0.62 ± 0.02 | 0.51 ± 0.07* | 0.75 ± 0.11 | 0.23 ± 0.04 | 3.40 ± 0.22 | 0.14 ± 0.02 | 0.74 ± 0.09 | 0.22 ± 0.03 | 0.21 ± 0.03 | 0.43 ± 0.06 | 8.72 ± 0.84 | 2.10 ± 0.13 |
| | methyl jasmonate | 3.32 ± 0.22 | 0.66 ± 0.12 | 0.23 ± 0.06 | 1.19 ± 0.11* | 0.86 ± 0.12* | 3.99 ± 0.29* | 0.14 ± 0.02 | 4.82 ± 0.87* | 0.22 ± 0.03 | 0.22 ± 0.03 | 0.44 ± 0.07 | 13.8 ± 1.28* | 2.00 ± 0.11 |
| Expo | control | 3.44 ± 0.20 | 1.09 ± 0.05 | 1.12 ± 0.07 | 2.22 ± 0.15 | 0.15 ± 0.01 | 2.81 ± 0.30 | 0.51 ± 0.08 | 1.50 ± 0.07 | 0.99 ± 0.15 | 0.52 ± 0.08 | 1.51 ± 0.23 | 11.5 ± 0.61 | 3.07 ± 0.21 |
| | methyl jasmonate | 5.21 ± 0.26* | 2.13 ± 0.30* | 1.30 ± 0.25 | 4.50 ± 0.47* | 1.96 ± 0.88* | 4.33 ± 0.28* | 0.57 ± 0.09 | 14.7 ± 0.60* | 3.67 ± 0.55* | 1.03 ± 0.15* | 4.70 ± 0.71* | 30.6 ± 1.49* | 3.31 ± 0.13 |
| Green Magic | control | 4.36 ± 0.16 | 1.07 ± 0.21 | 1.22 ± 0.37 | 1.11 ± 0.02 | 0.54 ± 0.04 | 2.91 ± 0.53 | 0.21 ± 0.03 | 3.06 ± 0.62 | 0.76 ± 0.11 | 0.36 ± 0.05 | 1.11 ± 0.17 | 13.8 ± 1.61 | 2.81 ± 0.24 |
| | methyl jasmonate | 4.25 ± 1.28 | 1.40 ± 0.11 | 0.85 ± 0.10 | 1.92 ± 0.11* | 1.25 ± 0.18* | 2.45 ± 0.17 | 0.26 ± 0.04 | 11.7 ± 0.55* | 2.00 ± 0.30* | 1.55 ± 0.23* | 3.55 ± 0.53* | 22.3 ± 1.24* | 3.06 ± 0.09 |
| Imperial | control | 3.64 ± 1.01 | 1.16 ± 0.13 | 0.80 ± 0.22* | 1.60 ± 0.10 | 0.45 ± 0.21 | 3.44 ± 0.22 | 0.23 ± 0.03 | 2.02 ± 0.40 | 0.45 ± 0.07 | 0.21 ± 0.03 | 0.65 ± 0.10 | 11.5 ± 1.40 | 2.80 ± 0.01 |
| | methyl jasmonate | 2.67 ± 0.61 | 1.02 ± 0.14 | 0.16 ± 0.06 | 2.22 ± 0.77 | 1.44 ± 052* | 3.09 ± 1.16 | 0.46 ± 0.16 | 12.2 ± 4.56* | 1.59 ± 0.56* | 0.98 ± 0.34* | 2.56 ± 0.90* | 20.4 ± 7.09 | 2.95 ± 0.09* |
| Gypsy | control | 3.94 ± 0.26 | 1.16 ± 0.18 | 0.34 ± 0.04 | 1.18 ± 0.38 | 0.23 ± 0.20 | 1.96 ± 0.62 | 0.21 ± 0.03 | 2.56 ± 1.34 | 0.86 ± 0.13 | 0.23 ± 0.04 | 1.09 ± 0.16 | 10.3 ± 2.46 | 2.47 ± 0.06 |
| | methyl jasmonate | 4.57 ± 1.30 | 1.25 ± 0.12 | 0.32 ± 0.16 | 2.21 ± 0.93 | 0.86 ± 0.40* | 2.51 ± 1.06 | 0.26 ± 0.04 | 10.7 ± 2.90* | 1.75 ± 0.61 | 0.69 ± 0.10* | 2.44 ± 0.37* | 20.6 ± 6.38 | 2.83 ± 0.08* |
| genotypes 2010 | | | | | | | | | | | | | | |
| Pirate | control | 2.93 ± 1.03 | 1.36 ± 0.18 | 2.79 ± 0.92 | 2.34 ± 0.94 | 0.50 ± 0.09 | 3.48 ± 1.09 | 0.27 ± 0.04 | 4.86 ± 1.83 | 0.81 ± 0.12 | 2.31 ± 0.12 | 3.13 ± 0.47 | 16.6 ± 5.76 | 3.86 ± 0.27 |
| | methyl jasmonate | 2.63 ± 0.59 | 1.77 ± 0.26 | 2.16 ± 0.37 | 2.07 ± 1.15 | 0.80 ± 0.43 | 2.68 ± 0.38 | 0.39 ± 0.06* | 5.12 ± 3.41 | 1.27 ± 0.19* | 1.74 ± 0.19 | 3.01 ± 0.45 | 14.8 ± 5.92 | 4.21 ± 0.12 |
| Expo | control | 3.62 ± 0.68 | 1.59 ± 0.36 | 2.13 ± 0.63 | 2.98 ± 0.14 | 0.65 ± 0.33 | 2.37 ± 0.39 | 0.39 ± 0.06 | 4.83 ± 0.72 | 1.36 ± 0.34 | 1.96 ± 0.34 | 3.32 ± 0.50 | 16.5 ± 0.91 | 4.08 ± 0.24 |
| | methyl jasmonate | 3.73 ± 0.53 | 1.60 ± 0.15 | 1.57 ± 0.18 | 3.31 ± 0.36 | 1.21 ± 0.20 | 1.74 ± 0.37 | 0.35 ± 0.05 | 7.14 ± 0.80* | 1.94 ± 0.49 | 3.52 ± 0.49* | 5.46 ± 0.82* | 18.1 ± 1.84 | 4.41 ± 0.25 |
| Green Magic | control | 1.82 ± 0.35 | 0.50 ± 0.08 | 1.91 ± 0.39 | 1.24 ± 0.01 | 0.30 ± 0.02 | 2.41 ± 0.28 | 0.23 ± 0.03 | 3.63 ± 0.09 | 0.55 ± 0.08 | 1.31 ± 0.08 | 1.86 ± 0.28 | 11.6 ± 0.97 | 3.32 ± 0.10 |
| | methyl jasmonate | 2.76 ± 0.42* | 1.04 ± 0.09* | 1.30 ± 0.04 | 2.19 ± 0.18* | 0.58 ± 0.08* | 1.94 ± 0.37 | 0.27 ± 0.04 | 10.7 ± 2.12* | 2.33 ± 0.35* | 5.17 ± 0.35* | 7.50 ± 1.13* | 19.6 ± 2.11* | 3.86 ± 0.24* |
| Imperial | control | 4.54 ± 1.08 | 1.63 ± 0.41 | 3.64 ± 0.71* | 1.67 ± 0.19 | 0.23 ± 0.06 | 2.54 ± 0.17 | 0.16 ± 0.02 | 3.01 ± 0.23 | 0.42 ± 0.06 | 1.19 ± 0.06 | 1.60 ± 0.24 | 15.5 ± 1.03 | 3.75 ± 0.13 |
| | methyl jasmonate | 6.07 ± 0.88 | 2.47 ± 0.32* | 1.92 ± 0.30 | 3.69 ± 0.52* | 1.42 ± 0.19* | 1.90 ± 0.42 | 0.30 ± 0.05* | 11.7 ± 2.862010 | 3.29 ± 0.49* | 4.29 ± 0.49* | 7.59 ± 1.14* | 25.4 ± 4.46* | 4.31 ± 0.09* |
| Gypsy | control | 8.70 ± 1.03 | 0.66 ± 0.30 | 2.79 ± 0.92 | 1.71 ± 0.21 | 0.23 ± 0.08 | 1.37 ± 0.19 | 0.17 ± 0.03 | 2.65 ± 0.15 | 0.40 ± 0.06 | 1.16 ± 0.06 | 1.56 ± 0.23 | 19.9 ± 1.93 | 3.74 ± 0.21 |
| | methyl jasmonate | 8.99 ± 0.85 | 3.08 ± 0.09* | 2.16 ± 0.37 | 3.55 ± 0.88* | 1.54 ± 0.71* | 1.71 ± 0.22 | 0.20 ± 0.03 | 11.6 ± 1.38* | 3.23 ± 0.49* | 2.93 ± 0.49* | 6.16 ± 0.92* | 29.4 ± 4.08* | 4.36 ± 0.07* |

[a]Values represented as **4b** molar equivalent concentrations (μmol per g of DW). [b]Total GS = total glucosinolate, including presented glucosinolates in table and glucoiberin and proitrin. [c]Asterisk symbol indicates that MeJA treatment is significantly different based on Student's *t* test at $P = 0.05$.



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shift toward increasing **5a** biosynthesis. Individual cultivars responded differently to methyl jasmonate treatment, with 'Pirate' showing no significant increase in any GS in 2010 while the other four hybrids displayed significantly increased concentrations of **3a** and **5a**. Since the 'Pirate' cultivar has a late maturity (Table 1), insect activity prior to harvest might have upregulated GS biosynthesis, making the plants less responsive to exogenous methyl jasmonate treatment. There was significant year-to-year variation in total GS. Total GS in control broccoli lines grown in 2010 was 43% higher than in controls grown in 2009. Precipitation in August 2010 (40 mm) was only 29% of that observed in August 2009 (137 mm). This observation agrees with the finding that water stress can increase total GS, as has been previously reported for *B. napus*.[38] Average temperatures in August 2010 were 17% higher than in August 2009. It has also been suggested that increased temperatures can result in the accumulation of GS by upregulating myeloblastosis (MYB) transcription factors as was observed in turnips.[39]

**Effect of Methyl Jasmonate Treatment on GS Hydrolysis Product Concentrations and QR Inducing Activity.** **5b2**, a hydrolysis product of **5a**, was significantly increased by methyl jasmonate treatment in four cultivars over two seasons with the exception of 'Pirate'. **5b1** and **5b2** concentrations in hydrolyzed 'Pirate' floret extracts were not increased in 2009 or 2010 by methyl jasmonate treatment (Table 2). Across all genotypes **5b2** concentrations in 2009 controls were significantly higher than in 2010. Endogenous JA biosynthesis is responsive to many biotic and abiotic stresses, including drought stress.[40] Exogenous methyl jasmonate treatments have been observed to increase ascorbic acid concentration in broccoli florets, *Arabidopsis*, and tobacco BY-2 cell suspension cultures.[41,42] Drought stress conditions in 2010 may have led to production of more **5b2** since there was a greater abundance of both substrates (**5a** and ascorbic acid). **1b** (control:methyl jasmonate = 31%:36%) and **3b** (control:methyl jasmonate = 28%:51%) conversion rates (averaged value) from their parent GS were also significantly increased by methyl jasmonate treatment (Table 3). However, the effect was not consistently observed in all cultivars over both years. This increased isothiocyanate formation by methyl jasmonate treatment may be involved in herbivore defense.[43] Methyl jasmonate treatment is known to increase gene expression of broccoli myrosinase.[44] The unbound myrosinase free of the cofactor, epithiospecifier protein (ESP), favors the generation of isothiocyanates instead of QR inactive nitrile forms.[45] In addition, MeJA treatment also might have altered the ratios of ESP and epithiospecifier modifier protein 1 (ESM1) myrosinase cofactors resulting in enhanced isothiocyanate formation.[46] This proposed mechanism for increased isothiocyanate concentrations is also supported by our gene expression data of myrosinase and its cofactors.[47] Although glucobrassicin was not significantly increased both years by methyl jasmonate treatment, **4b** concentration was significantly increased in both 2009 and 2010.

Based on our time course experiments, aliquots were taken after 4 h of broccoli extract hydrolysis for measurement of QR inducing activities. Treatment with methyl jasmonate significantly increased QR inducing activity of extracts from florets of 'Imperial' and 'Gypsy' cultivars in both years. Average QR inducing activity across all of the five broccoli genotypes was significantly increased by methyl jasmonate treatment only in 2010, which suggests an interaction between methyl jasmonate treatment and year.

**Table 3. Isothiocyanate Conversion Efficiency (%) from Precursor GS into 1b and 3b**

| source of variation | treatment | **1b**/**1a** (%) | **3b**/**3a** (%) |
|---|---|---|---|
| treatment | | | |
| | control | 30.7 ± 11.5 | 27.8 ± 19.9 |
| | methyl jasmonate | 35.5 ± 12.4*[a] | 50.8 ± 19.2* |
| year | | | |
| 2009 | control | 29.1 ± 6.5 | 36.2 ± 24.7 |
| | methyl jasmonate | 30.3 ± 10.1 | 64.6 ± 15.5* |
| 2010 | control | 32.2 ± 13.6 | 19.3 ± 7.4 |
| | methyl jasmonate | 40.7 ± 10.4* | 36.9 ± 10.5* |
| genotypes 2009 | | | |
| Pirate | control | 20.6 ± 2.7 | 31.9 ± 8.9 |
| | methyl jasmonate | 20.0 ± 5.1 | 72.1 ± 7.7* |
| Expo | control | 31.7 ± 2.5 | 6.9 ± 0.6 |
| | methyl jasmonate | 40.1 ± 5.5* | 44.7 ± 21.5* |
| Green Magic | control | 32.0 ± 5.1 | 48.6 ± 4.3 |
| | methyl jasmonate | 25.1 ± 10.3 | 64.9 ± 7.6* |
| Imperial | control | 31.9 ± 11.6 | 28.3 ± 13.3 |
| | methyl jasmonate | 38.0 ± 6.0 | 64.9 ± 4.4* |
| Gypsy | control | 29.4 ± 4.0 | 65.5 ± 32.6 |
| | methyl jasmonate | 27.4 ± 11.5 | 76.4 ± 14.0 |
| genotypes 2010 | | | |
| Pirate | control | 46.5 ± 8.8 | 22.7 ± 6.8 |
| | methyl jasmonate | 67.2 ± 8.4* | 41.0 ± 17.2 |
| Expo | control | 44.1 ± 12.7 | 21.8 ± 10.9 |
| | methyl jasmonate | 43.1 ± 6.1 | 36.6 ± 5.4 |
| Green Magic | control | 27.6 ± 5.7 | 24.3 ± 1.2 |
| | methyl jasmonate | 18.2 ± 10.6 | 26.3 ± 2.0 |
| Imperial | control | 35.9 ± 6.0 | 14.0 ± 5.6 |
| | methyl jasmonate | 40.8 ± 8.3 | 38.6 ± 1.9* |
| Gypsy | control | 7.6 ± 3.4 | 13.7 ± 5.5 |
| | methyl jasmonate | 34.2 ± 3.7* | 42.1 ± 14.3* |

[a]Asterisk symbol indicates that MeJA treatment is significantly different based on Student's $t$ test at $P = 0.05$.

**Correlation Analysis between Intact GS or Hydrolysis Products and QR Activity.** There was a moderate but significant correlation between **3a** and QR inducing activity where $r = 0.654$ ($P = 0.002$). For the precursor GS, there were only weak and nonsignificant correlations between **1a** ($r = 0.330$; $P = 0.155$), **5a** ($r = 0.312$ $P = 0.181$), and QR inducing activity. **3b**[12] and **1b**[5] are known and potent QR inducers. The moderate correlation coefficient between **1a** and **1b** concentrations ($r = 0.593$, $P = 0.006$) among the five broccoli cultivars may be associated with variation in ESP and/or ESM1 activity, since **1b** formation is negatively correlated with epithiospecifier protein levels.[45] Although there was a significant positive correlation between **3a** and QR, there was a nonsignificant correlation between **3b** and QR inducing activity ($r = 0.176$, $P = 0.459$). This lack of correlation is likely due to the high volatility and low solubility of **3b**, resulting in low accumulation in broccoli extracts. The observed moderate correlation between **3a** and QR inducing activity may be due to the correlation of **3a** with **1b** ($r = 0.784$, $P < 0.001$), **5b1** ($r = 0.876$, $P < 0.001$), and **5b2** ($r = 0.549$, $P = 0.012$).

There were significant correlations between QR activity and the hydrolysis products of **5a** including **5b1** ($r = 0.502$, $P = 0.024$) and **5b2** ($r = 0.771$, $P < 0.001$). Recent research using


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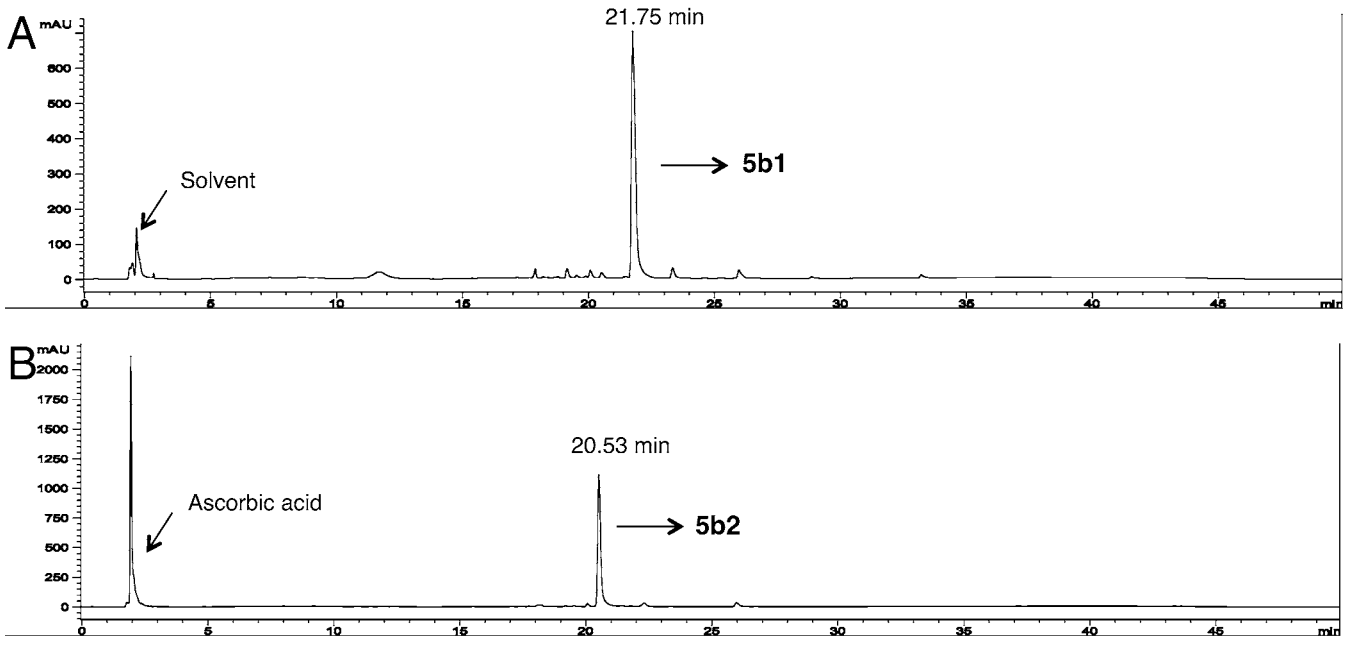


**Figure 3.** Confirmation of major hydrolysis products of **5a** to measure QR inducing activity.

an in vitro cancer cell line has found that **5b1** can inhibit the nuclear erythroid related factor 2 (Nrf2)-dependent upregulation of phase II detoxifying enzymes such as QR, interfering with its anticancer bioactivity.[48] This suggests that methyl jasmonate-mediated upregulation of **5a** biosynthesis in broccoli florets would interfere with the QR induction associated with the **1b**. However, there was a significant positive correlation between **1b** and QR activity when **5b1** was present at the highest concentrations in the 4 h hydrolysis extracts, indicating it did not inhibit **1b** dependent increases in QR activity. This result implies that concentration of **5b1** in methyl jasmonate treated broccoli is not at a critical level to interfere with **1b** mediated QR activity induction. A previous study[48] used in situ hydrolysis for QR activity measurement, which favors the production of **5b1** from **5a** using neutral pH. This experiment did not consider the formation of other hydrolysis products that could be generated from neoglucobrassicin. In our study **5a** was observed to generate both **5b1** and **5b2**, which is the condensation product of **5b1** with ascorbic acid (Figure 3). **5b2** may be the primary hydrolysis product from **5a** in the human gut where low pH and high vitamin C concentrations[49] from broccoli consumption could favor the production of **5b2** over **5b1**.

**QR Inducers in Methyl Jasmonate Treated Broccoli.** High correlation of QR activity with **5b1** and **5b2** suggests that the hydrolysis products of **5a** may contribute to the methyl jasmonate enhanced QR activity. However, the concentrations required to double QR induction values of each compound were found to be 35 $\mu$M and 38 $\mu$M for **5b1**and **5b2**, respectively. Previously it was reported that the concentrations required to double QR induction activity of **3b** and **1b** were 5 $\mu$M and 0.2 $\mu$M, respectively,[18,50] indicating that **5b1** and **5b2** are relatively weak QR inducing agents. Averaged concentration required to double QR induction value of the hydrolysis products of **5a** is approximately 36.5 $\mu$M. When the QR induction is evaluated by the relative magnitude of estimated concentration required to double QR induction values and relative concentrations of hydrolysis products, **1b** should possess a 38-fold greater QR inducing capacity than the hydrolysis products of **5a**.

**Effect of Weather Related Factors on Glucosinolates, Their Hydrolysis Products, and QR Activity.** GDD accumulation among the cultivars and seasons was significantly correlated with **2** ($r$ = 0.634, $P$ = 0.020), **5b2** ($r$ = 0.496, $P$ = 0.026), and QR inducing activity ($r$ = 0.699, $P$ < 0.001). Accumulated solar radiation was significantly correlated with **2** ($r$ = 0.634, $P$ = 0.003), **5b2** ($r$ = 0.586, $P$ = 0.007), and QR inducing activity ($r$ = 0.796, $P$ < 0.001). PPT/DTH was significant negatively correlated with **1b** ($r$ = −0.447, $P$ = 0.048) and QR inducing activity ($r$ = −0.660, $P$ = 0.002), suggesting that drought conditions may enhance QR inducing activity by enhancing endogenous JA synthesis.[40]

DTH of broccoli cultivars used in this experiment was significantly different among genotypes ($F_{4,19}$ = 18.32, $P$ < 0.001) (Table 1). The partitioning of total variance into variance components indicated that differences among genotypes account for 75% of DTH variation, which agrees with previous research.[51] Since DTH is correlated with solar radiation and GDD, genetic variation in days to harvest plays a major role in broccoli floret phytochemical composition and QR inducing activity. QR activity is indirectly affected by weather, DTH, and GDD.

**Partitioning of GS Concentration and Bioactivity Variances into Methyl Jasmonate Treatment, Year, and Genotype Sources of Variation.** ANOVA partitioning of the variances for GS concentrations indicated that differences among genotypes described 48%, 42%, 33%, and 31% of the total variation for **4b**, **1a**, **4a**, and **3a** respectively. In contrast, methyl jasmonate treatment accounted for 63%, 46%, 36%, 30%, and 17% of the total variation in floret **5a**, **5a1**, total GS, **3b**, and **1b** concentrations, respectively. Seasonal differences in environmental conditions between 2009 and 2010 were a major source of variation in QR inducing activity (72%), **5b2** (48%), and **1b** (29%). There was also a significant genotype by year interaction in concentrations of **1a** (38%) and **4b** (23%). Methyl jasmonate treatment significantly increased QR inducing activity averaged over the two year study, but this only described 5% of


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the variation in QR inducing activity while year associated weather effects accounted for 72%, which also agrees with previous research.[51]

In conclusion, treatment with methyl jasmonate significantly increased **1a**, **3a**, and **5a** concentrations in florets across five broccoli hybrids, over two seasons, under field conditions. Concentrations of **1b** and hydrolysis products of **5a** were significantly and positively correlated with enhanced QR inducing activity. This present study found that methyl jasmonate treatments not only increase GS biosynthesis but also increase isothiocyanate formation from GS precursors. Among increased hydrolysis products by methyl jasmonate treatment, **1b** is a major contributor toward enhanced QR induction activity in broccoli floret extracts, although other GS and their hydrolysis products are also likely contributors. Environment conditions significantly correlated with GS biosynthesis and hydrolysis products of GS. These results suggest that optimal environment conditions, appropriate cultivars, and methyl jasmonate treatments can maximize phytochemical profiles and anticancer bioactivity of broccoli florets.

## ■ ASSOCIATED CONTENT

### Supporting Information

Supplementary Table 1, weather information for 2009 and 2010 in Champaign, Illinois. Supplementary Table 2, tentative identification of purified hydrolysis products of **5a**. Supplementary Table 3, correlations among GS and hydrolysis product concentrations, QR inducing activities and weather related variables. Supplementary Table 4, percentages of total variance described by main factors (genotype, treatment, and year) and factor interactions for broccoli floret phytochemical concentrations and bioactivities. Supplementary Figure 1, relative QR activity of 30 min and 4 h hydrolyzed extracts to 24 h hydrolyzed extracts. Supplementary Figure 2, the concentration required to double QR induction values of hydrolysis products of **5a**. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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