Changes in "Natural Antibiotic" Metabolite Composition During Tetraploid Wheat Domestication

Yuval Ben-Abu (✉ yuvalb@sapir.ac.il)

Sapir College

Mark Itsko

Centers for Disease Control and Prevention

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Abstract

Plants defend themselves by producing toxins and deterrent metabolites. However, it is unclear how the composition and abundance of these natural pesticides have changed over the course of crop-plant domestication. To address this uncertainty, we characterized differences in secondary metabolites, particularly Benzoxazinoids and its derivatives, among four lines of tetraploid wheat: wild emmer wheat (WEW), the direct progenitor of modern wheat; non-fragile domesticated emmer wheat (DEW), which was first domesticated about 11,000 years ago; the subsequently developed non-fragile and free-threshing durum landraces (LD); and modern durum (MD) varieties. Mass spectrometry analyses showed that the metabolome of the embryo of the mature kernel was more complex than that of the endosperm. Clear differences were observed among the metabolic profiles of WEW, DEW, and durum (LD + MD); the metabolic profiles of the two durum lines (LD and MD) were similar. Our results indicated that underappreciated classes of metabolites involved in plant defense mechanisms became significantly more abundant during wheat domestication, while other defensive metabolites decreased or were lost. It may be that the use of industrial pesticides has led to the loss of these endogenous defense metabolites. The re-expression of such “lost” metabolites in modern wheat might help to improve crop resistance, while reducing dependence on harmful industrial pesticides. In addition, we detected alterations in antioxidant composition among wheat lines, as well as increases in levels of plant hormones and antibiotic substances in the more modern lines as compared to the more primitive lines. Here, we show how DIMBOA and its derivatives change during wheat domestication, and how this change may effect on the domestication of three taxonomic subspecies of tetraploid wheat (*Triticum turgidum*).

Introduction

Plants such as wheat (*Triticum aestivum*), rye (*Secale cereale*), and maize (*Zea mays*) defend themselves against bacteria, fungi, insects, or herbivores by producing toxins and deterrent metabolites such as benzoxazinones (Bxs), which are cyclic hydroxamic acids derived from indole [1] [2]. The hydroxamic acids are a family of secondary metabolites of cereals that were discovered over four decades ago in a relative of rye (Fig. 1.). Benzoxazinones are important secondary metabolites in gramineous plants and are one of the most investigated hydroxamic acids in the family *Poaceae* [1] [2] [3] [4] [5] [6] [7]. Benzoxazinones are natural antibiotics and also play a major role in plant defense against fungi [8] [9] [6], insects [10], and weeds [11]. The most abundant benzoxazinones are 2,4-dihydroxy-1,4-benzoxazin-3-one (DI-BOA) and its 7-methoxy analog (DIMBOA), which are constitutively present in the vacuole as glucosides (DIBOA-Glc and DIMBOA-Glc) [12, 6] [13]. Bx-glucosides are particularly important for plant defense during the juvenile stage [15].

Benzoxazinone composition and abundance change throughout plant life cycle. For example, Bx-glucoside abundance is greatest soon after germination, decreasing thereafter to low levels [15]. Relative benzoxazinone abundance has also been shown to vary among plant crops (e.g., maize and wheat [21]), tissues (e.g., shoots and leaves [6]), growth habitats (e.g., arid and humid [6]), and even soil type (e.g., dry

and wet [22]). In addition, dramatic differences in benzoxazinone content have been identified in microscale evolutionary contexts [22].

Using three taxonomic subspecies of tetraploid wheat (*Triticum turgidum*), we aimed to clarify how benzoxazinones were affected by plant domestication and how this change may effect on the what domestication. Tetraploid wheat, specifically wild emmer wheat (WEW; *Triticum turgidum* ssp. *dicoccoides*), was first domesticated about 11,000 years ago (Fig. 2) [16]. Although domesticated emmer wheat (DEW; *T. turgidum* ssp. *dicoccum*, genome BBAA, 4x, 2n = 28) was widely cultivated for several millennia, today it exists only as a relatively minor crop, having been replaced, mostly during the Roman period, by durum wheat (*T. turgidum* ssp. *durum*, genome BBAA, 4x, 2n = 28), which is non-fragile and free-threshing [16] [17]. Domesticated durum varieties can be divided into ancient durum landraces (LD), locally adapted varieties that were selected by farmers and grown using low input farming practices [18], and modern durum (MD) varieties, including both dwarf and semi-dwarf lines, which were developed by plant breeders and which are grown on modern farms [CITE]. These four wheat lines represent both primary domestication (WEW to DEW) and secondary domestication (DEW to LD/WD) [24]. The durum LD and MD lines differ both genetically and phenotypically; we considered these lines separately in order to capture changes that occurred during the green revolution, when traditional landraces transitioned to modern varieties [24]. Hexaploid bread wheat (*Triticum aestivum* ssp. *aestivum*, genome BBAADD, 6x, 2n = 42) was developed in the fertile crescent ~ 9,000 years ago by hybridizing tetraploid wheat (genome BBAA) with a diploid grass, *Aegilops tauschii* (genome DD, 2x, 2n = 14), followed by whole-genome doubling [19] [20]. Bread wheat spread from the fertile crescent to many different environments and is at present the most widely grown wheat crop [20].

In this study, we focused on domestication-associated changes in the composition and abundance of benzoxazinone and its derivatives in the four lines of tetraploid wheat. Using mass spectrometry (MS), we found that the metabolome of the embryo of the mature kernel was much more complex than that of the endosperm. We then examined how domestication affected metabolite profiles by comparing modern wheat (MD) to its progenitors. To specifically investigate changes in the relative abundances of benzoxazinone and its derivatives during wheat domestication, we performed an MS-coupled metabolomic analysis of the kernel embryo and endosperm to separate benzoxazinone compounds and their derivatives. Our analyses revealed dramatic changes in benzoxazinone composition over the course of wheat domestication, as evidenced by distinct separations among WEW, DEW, and durum wheat; benzoxazinone profiles were similar between LD and MD. This remarkable separation among WEW, DEW, and durum highlighted the effects of domestication on benzoxazinone components. It may be that the use of industrial pesticides, climate change, or shifts in growth habitat have led to alterations in the expression patterns of these endogenous benzoxazinones. Such “lost” benzoxazinones might be valuable for improving crop resistance, while minimizing dependence on harmful industrial pesticides. Both biotic stress (e.g., pathogens) and abiotic stress (e.g., lack of nutrients, extreme temperatures and drought) may lead to oxidative stress in plants, against which antioxidants can provide protection. Thus, our results may provide a reference for the identification of optimal breeding strains with pre-domestication benzoxazinone profiles or of novel genes encoding for certain benzoxazinones “lost”

during domestication, the incorporation of which may improve wheat nutritional value or pest resistance. our hypothesis that metabolite composition and expression underwent substantial changes during wheat domestication.

Materials And Methods

Plant material

The study complies with local and national regulation. Nineteen accessions representing a total of four wheat lines corresponding to various stages of tetraploid wheat domestication were included in this study: five WEW accessions (*T. turgidum* ssp. *dicoccoides*), five DEW accessions, (*T. turgidum* ssp. *dicoccum*), five LD accessions (*T. turgidum* ssp. *durum*), and four MD accessions (*Triticum turgidum* ssp. *durum*; Table 1). Different wheat types were originally collected from traditional farmers from different country as described in Table 1. All plants were grown in individual 3 L pots in a net house under identical conditions. Three replicates of each accession were grown, and each replicate was cultivated in a separate block.

Table 1

Tetraploid wheat lines used in this study. Country of origin for each line is shown in parentheses.

Wild Emmer wheat (WEW) <i>Triticum turgidum</i> ssp. <i>Dicoccoides</i>	Domestic Emmer wheat (DEW) <i>Triticum turgidum</i> ssp. <i>dicoccum</i>	Landraces Durum (LD) <i>Triticum turgidum</i> ssp. <i>durum</i>	Modern Durum (MD) <i>Triticum turgidum</i> ssp. <i>durum</i>
TTD20 (Israel)	Sub-group A: TTC1 (Italy)	TTR265 (Israel)	TTR16 (USA)
TTD32 (Turkey)	Sub-group A: TTC2 (India)	TTR2 (Israel)	TTR19 (Italy)
TTD43 (Syria)	Sub-group A: TTC8 (India)	TTR333 (Turkey)	TTR1 (Portugal)
TTD49 (Israel)	Sub-group B: TTC4 (Israel)	TTR5 (Morroco and Tunisia)	TTR25 (Israel)
TTD68 (Israel)	Sub-group B: TTC6 (Spain)	TTR6 (Israel)	

Sample preparation

Each whole grain sample was separated into endosperm and embryo, and metabolite extraction was performed as described previously [23]. In brief, the embryo and endosperm tissues (500 mg each) were separately ground to a fine powder in liquid nitrogen, and 1.5 mL of 75% methanol with 0.1% formic acid was added. After sonication at room temperature for 15 min, each sample was centrifuged at 10,000 · g and filtered. The filtered samples were stored at -20°C prior to analysis. The metabolites in the extractable

fractions were further purified using column chromatography (Amberlite XAD 8 HP) and eluted with ethanol. Both fractions were freeze-dried and stored at -80°C.

LC-MS metabolite analysis

Metabolite analysis was carried out using ultra-performance liquid chromatography coupled with a photodiode detector-quadrupole and tandem time-of-flight mass spectrometry (UPLC-PDA-qTOF-MS). The system consisted of a Acquity UPLC (Waters) connected in-line to an Acquity PDA detector (Waters) and a Synapt HDMS detector (Waters). The HDMS system was operated in the standard qTOF mode, without using the ion mobility capabilities. Metabolite separation was performed using a UPLC BEH C18 column (100 · 2.1 mm i.d., 1.7 µm; Waters). The mobile phase consisted of 0.1% formic acid in acetonitrile/water (5:95, v/v) (phase A) and 0.1% formic acid in acetonitrile (phase B). The linear gradient program was as follows: 100–72% A over 22 min, 72–60% A over 0.5 min, 60–0% A over 0.5 min, holding at 100% B for a 1.5 min, then returning to initial conditions (100% A) over 0.5 min, and conditioning at 100% A. The flow rate was 0.3 mL/min, and the column temperature was kept at 35°C. The UV spectra were recorded at 210–550 nm using the Acquity PDA detector (Waters), or the UV trace was measured at 240 nm using the Acquity UV detector (Waters). Eluting compounds were detected using the qTOF equipped with an electrospray ionization (ESI) source. Acquisition was performed in ESI-positive and ESI-negative modes. The following settings were applied during the LC-MS runs: capillary voltage, 3.0 kV; cone voltage, 30 eV; collision energy, 3 eV and 20 eV; and collision gas, argon. For the LCMS/MS analysis, collision energies of 20 and 35 eV were used. The m/z range was 50–1500 Da. The MS was calibrated using sodium formate, and leucine enkephalin was used as the lock mass. A standard mixture containing 40 µg/mL of each of the following compounds was used to monitor the quality of the chromatogram, to ensure the consistency of retention times across runs, and to aid in metabolite identification: L-tryptophan, L-phenylalanine, p-coumaric acid, caffeic acid, sinapic acid, benzoic acid, quercetin dehydrate, kaempferol, rutin, and trans-resveratrol (all purchased from Sigma); naringenin, chlorogenic acid hemihydrate, trans-cinnamic acid, and isorhamnetin (Fluka); ferulic acid (Aldrich); and tomatine (Apin chemicals). Mass Lynx v4.1 (Waters) was used to control all instruments and to calculate accurate masses.

LC-MS data analysis

The chromatograms obtained using UPLC PDA-qTOF-MS analysis were processed using Marker Lynx v4.1 (Waters) to extract and align mass signals. Metabolite identification was performed as described previously on data obtained using the ESI-negative mode [27]. In brief, accurate mass and molecular formula predictions for the putative molecules were screened against the Dictionary of Natural Products (Chapman and Hall/CRC) and the SciFinder Scholar databases (SciFinder Scholar 2007). The MS/MS fragmentation and UV-absorption of the metabolites were compared with those of candidate molecules found in databases and verified using previously published studies of similar compounds.

Statistical analyses

Statistical analyses of the datasets were performed using Microsoft Excel 365 and MATLAB 8.0 with Statistics Toolbox 8.1. P-values (FDR) < 0.05 were considered significant. We ascribed functions to

metabolites based on searches of KEGG and HMDB, as well as complementary internet searches. Figures were generated using MATLAB 8.0, GraphPad Prism 7.0, and Meta-Chart.

Results

Domestication-associated metabolomic alterations in wheat lines

We first compared metabolomes between the embryo and endosperm of 19 accessions, representing three wild and domesticated tetraploid wheat subspecies at various stages of domestication and from a variety of eco-geographical locations (Table 1). Across all accessions, LC-MS/MS analyses detected 4886 distinct metabolites in the embryo samples and 413 metabolites in the endosperm samples, showing that the embryo metabolome was much more complex than the endosperm metabolome. Next, we generated metabolite profiles for each accession by averaging metabolite values across replicas and normalizing the distributions. Tests of group effects identified nine unique metabolites in the embryo samples and 154 unique metabolites in the endosperm samples (data not shown), again suggesting that the embryo metabolome was far more complex than the endosperm metabolome. Heatmaps of upregulated and downregulated metabolites in the embryo and the endosperm (Fig. 3A–B) revealed global patterns of metabolomic differences among the four wheat lines as well as consistent differences within lines. The dendrogram representing hierarchical clustering of the embryo metabolome heatmap showed distinct separations among the WEW, DEW, and durum lines (Fig. 3C). Interestingly, the durum accessions were divided into two subgroups, which did not correspond to LD and MD; the DEW accessions were also divided into two subgroups (Fig. 3C).

Three-dimensional (3D) principal component analyses (PCAs), run without a wheat category-bias, also demonstrated a clear separation among the WEW, DEW, and durum accessions in both the embryo and the endosperm tissues (Fig. 3). Similar to the heatmaps, the 3D PCA models did not distinguish between the MD and LD accessions (Fig. 3). We again observed two distinct subgroups within the DEW accessions (Fig. 3). These DEW subgroups were designated A and B (Table 1). Thus, the heatmaps and the PCAs were consistent with our hypothesis that metabolite composition and expression underwent substantial changes during wheat domestication.

Identification of benzaxonoise metabolites associated with wheat domestication

To identify specific changes in benzaxonoise composition, we searched the metabolites present in the wheat kernels against an annotated database [25]. We identified nine benzaxonoise metabolites in the embryo samples and seven benzaxonoise metabolites in the endosperm samples. We then characterized the metabolites that underwent significant changes during wheat domestication based on the averaged metabolomes of each group. Because there was no clear separation between LD and MD, we considered

durum wheat a single group. We thus compared WEW to DEW, DEW to durum, and WEW to durum, with the WEW to durum comparison effectively summarizing the domestication process (Figs. 3 and 4).

Discussion

Bacteria, insects, fungi, and herbivores are frequently exposed to plant defense metabolites, either by coming into direct contact with plants or by preying on herbivores that have accumulated plant defense molecules [26] [27] [28]. Many natural enemies of wheat or other plants avoid plant defenses by rejecting herbivores that accumulate or sequester toxins [29] [30]. The toxicity levels of plant defense metabolites such as benzoxazinones may change substantially in different environments, even within a single plant species or cultivar [6]. Although the benzoxazinone hydroxamic acids have been well investigated, the effects of domestication on these secondary metabolites and their derivatives remain unclear [14]. Indeed, previous studies of kernel metabolite composition during tetraploid wheat domestication have focused primarily on primary metabolites [25], while studies of secondary metabolites have most frequently investigated the agronomic and environmental factors impacting these compounds [31]. Therefore, to our knowledge, no previous studies have characterized domestication-associated changes in the composition of secondary metabolites, particularly benzoxazinones. Our results showed that the relative abundances of several antibiotic benzoxazinoids were altered in the wheat kernel endosperm and embryo during domestication. Indeed, we identified several metabolites not previously recovered in the wheat metabolome, nor previously associated with domestication [25] [32].

Here, clear divergences among three stages of domestication (WEW, DEW, and LD/MD) were observed in the PCAs, heatmaps, and evolutionary dendrograms. No clear separation was observed between MD and LD, possibly due to the short time since the divergence of these groups (60–70 years; [CITE]) and/or a lack of selective pressure under modern farming practices. Importantly, our comparative MS-coupled metabolomics analysis revealed that the relative abundances of benzoxazinoid metabolite derivatives in the wheat kernel changed substantially during primary domestication (WEW to DEW) and secondary domestication (DEW to durum; Fig. 4). These shifts in composition and abundance may be due to climate change [44] [45], biotic stress, or other types of environmental alterations [6]. Levels of antibiotic substances were greater in different stages of wheat domestication, suggesting that some species during the wheat domestication survives and some disappear. Remarkably, DIMBOA and its derivatives showed significant activity during wheat domestication, may indicate to disappearance or survives according to changes in the response to biotic stressors (e.g., fungi and other pathogens).

More metabolites and more changes in metabolite composition were identified in the kernel embryo as compared to the endosperm, this mainly that the most substances in these tissues exist in the embryo. Notably, in the embryo, we detected greater levels of secondary metabolites associated with plant defense mechanisms and the response to biotic stress (Fig. 5), including phytoalexins, jasmonic acids, benzoxazinoids, glucosinolates, and other alkaloids that act as bactericides, fungicides and insecticides in various plants (Figs. 2,3 and 4) [33] [34] [35]. Specifically, benzoxazinoids were previously shown to participate in the stress response of WEW [6]. Actually, appearance or disappearance of such of these

Bzx, mainly in the embryo tissue that important for the next generation, may indicate about the resistance and survival of the plant [6]. Disease resistance is an adaptive trait that strongly affects crop productivity [36]. Interestingly, some defense-related metabolites (e.g., alkaloids) were downregulated in the MD lines as compared to the LD lines (Figs. 4 and 5). The use of industrial pesticides may have led to the downregulation of these endogenous pesticides [6]. The re-expression of such “lost” metabolites in modern lines might help to improve crop resistance, while minimizing dependence on harmful industrial pesticides [37]. Indeed, crop resilience might be most usefully improved by using genetic selection or engineering to increase the release of phytochemicals that confer resistance to biotic stress.

Both biotic stressors (e.g., pathogen invasion) and abiotic stressors (e.g., nutrient deficiencies, extreme temperatures, and drought) may lead to oxidative stress in plants, against which antioxidants can provide protection [38]. Genes that induce antioxidant expression in wheat may also confer disease resistance [39, 40]. In addition, high levels of antioxidants benefit the eventual consumers of wheat as a food source [41, 42, 43]. Our comparative metabolomics analysis revealed that antioxidants, including apigenin derivatives, flavonols, and glutathiones [38], were greater in the kernels of the domesticated wheat lines as compared to the wild wheat (i.e., WEW; Fig. 5).

Our results showed that benzoxazinoid production was substantially altered over the course of domestication (Fig. 4). It may be that the use of benzoxazinoids and their derivatives inhibited the expression of endogenous pesticides. Thus, our results may provide a reference for the identification of optimal breeding strains with pre-domestication benzoxazinone profiles or of novel genes encoding for certain benzoxazinones “lost” during domestication, the incorporation of which may improve wheat nutritional value or pest resistance.

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Figures

Figure 1

Schematic representation of the benzoxazinone (Bx) biosynthetic pathway, showing the enzymatic reactions catalyzed by the gene products of TaBx1–TaBx5 in the plastids and microsomes of tetraploid wheat.

Figure 2

Evolutionary history of allotetraploid and allohexaploid wheat: Diploid wheats ($2n=2X=14$), from the *Triticum-Aegilops* group have diverged $\sim 4\text{Mya}$ from a diploid progenitor whose genome is indicated here as PP. Intergeneric hybridization between the diploid *T. urartu* (genome AA) as male and the donor of BB genome as female, (an unknown species similar to *Ae. speltoides*), followed by chromosome doubling, gave rise ($\sim 0.5\text{Mya}$) to the wild allotetraploid wheat, *Triticum turgidum*, ssp. *dicoccoides* (genome BBAA, $2n=4x=28$), the direct progenitor of durum and bread wheat. Domestication of allotetraploid wheat took place $\sim 10,500$ years ago and was followed by a second round of intergeneric hybridization chromosome doubling between domesticated allotetraploid wheat and the donor of the D genome, *Ae. Tauschii* ($2n=2X=14$, genome DD), giving rise, ~ 9000 years ago, to bread wheat, an allohexaploid ($2n=6X=42$, genome AABBDD).

Figure 3

The embryo and endosperm metabolomes of 19 wheat accessions, representing three wild and domesticated tetraploid wheat subspecies at various stages of domestication: wild emmer wheat (WEW; *T. turgidum* ssp. *dicoccoides*), domesticated emmer wheat (DEW; *T. turgidum* ssp. *dicoccum*), durum landraces (LD; *T. turgidum* ssp. *durum*), and modern durum (MD; *Triticum turgidum* ssp. *durum*; see Table 1 for details of each accession). (A–B) Heat-maps of the (A) embryo and (B) endosperm metabolomes,

generated based on the quantile-normalized average values of metabolites per accession. Red metabolites were upregulated, and green metabolites were downregulated. (C) Dendrogram showing relationships among the wheat embryo metabolomes based on the hierarchical clustering patterns of the heat-map shown in (A). The longer the Euclidean distance between two accessions, the greater the difference between the respective metabolomes.

Figure 4

Three-dimensional models of principal component analyses (PCA) of the 19 wheat accessions, representing three wild and domesticated tetraploid wheat subspecies at various stages of

domestication: wild emmer wheat (WEW; *T. turgidum* ssp. *dicoccoides*), domesticated emmer wheat (DEW; *T. turgidum* ssp. *dicoccum*), durum landraces (LD; *T. turgidum* ssp. *durum*), and modern durum (MD; *Triticum turgidum* ssp. *durum*; see Table 1 for details of each accession). Each sphere represents the quantile-normalized metabolome of one replicate accession; well-separated groups are indicated with dashed lines. (A) Two views of the PCA of the embryo metabolomes. (B) Two views of the PCA of the endosperm metabolomes.

Figure 5

Changes in normalized benzoxazinone metabolites- natural antibiotic metabolites profile during tetraploid wheat domestication in the (A) endosperm and (B) embryo.