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The effects of GA and ABA treatments on metabolite profile of germinating barley

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ABSTRACT

Sugar degradation during grain germination is important for malt quality. In malting industry, gibberellin (GA) is frequently used for improvement of malting quality. In this study, the changes of metabolite profiles and starch-degrading enzymes during grain germination, and as affected by GA and abscisic acid (ABA) were investigated using two wild barley accessions XZ72 and XZ95. Totally fifty-two metabolites with known structures were detected and the change of metabolite during germination was time- and genotype dependent. Sugars and amino acids were the most dramatically changed compounds. Addition of GA enhanced the activities of starch-degrading enzymes, and increased most metabolites, especially sugars and amino acids, whereas ABA had the opposite effect. The effect varied with the barley accessions. The current study is the first attempt in investigating the effect of hormones on metabolite profiles in germinating barley grain, being helpful for identifying the factors affecting barley germination or malt quality.

1. Introduction

Barley is a most widely used cereal crop for brewing industry, and malting is an essential step for the production of beer and wines. During malting, the enzymes related to starch degradation are developed or activated. Under the coordinative actions of these starch-degrading enzymes, including α-amylase, β-amylase, limit dextrinase (LD) and α-glucosidase, starch as well as other polysaccharides in the endosperm of the germinating grains is degraded into monosaccharide, mainly glucose for further fermentation (Manners, 1974). Therefore high activities of these hydrolytic enzymes are favorable for complete degradation of starch and polysaccharides, leading to high malt extract, an important quality trait for malt barley.

It is commonly known that addition of GA could promote germination of barley grains during malting (Himmelbach, Iten, & Grill, 1998; Ritchie & Gilroy, 1998), which is attributed to enhanced synthesis and secretion of the enzymes related to seed germination (Bewley & Black, 1994). In the brewing industry, GA is frequently used for increasing activities of α-amylase and LD, thereby improving the malting quality (Chandler, Zwar, Jacobsen, Higgins, & Inglis, 1984; Hader, Rikiishi, Nisar, & Noda, 2003). On the other hand, ABA, as an antagonist of GA, inhibits seed germination (Frank, Scholz, Peter, & Engel, 2011). Chen and An (2006) used microarray analysis to investigate the transcriptional changes of barley aleurone in responses to GA and ABA treatments and detected more than 2200 genes, in which 1328 and 206 genes showed over three-fold change under GA or ABA treatment. However, little effort has been done to study the impact of GA or ABA treatment on metabolites including sugars, amino acids or organic acids in geminating barley grains.

The measurement of small metabolites has been facilitated by the development of gas chromatography–mass spectrometry (GC–MS) technology. Hence the metabolite profile of a single sample can be obtained, which may allow us to make insight into the metabolic processes in response to germinating conditions, such as addition of GA and ABA (Fiehn, 2002; Goodacre, Vaidyanathan, Dunn, Harrigan, & Kell, 2004). So far, GC–MS has been successfully applied in the studies on plant tolerance to abiotic stresses such as phosphate deficiency (Huang et al., 2008), salinity (Yousfi, Rabhi, Hessini, Abdelly, & Gharsalli, 2010) and drought (Guo et al., 2009). Frank et al. (2011) investigated the metabolites profiles of barley whole seeds during micro-malting, and Gorzolka, Lissel, Kessler, loch-ahring, and Niehaus (2012) conducted metabolomic analysis on barley malting at industrial scale.

In the modern barley breeding, narrower genetic diversity has become a bottleneck of developing the new cultivars with high

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biotic and abiotic stress tolerance, and better malt quality. Comparatively, wild barley is much wider in genetic diversity and regarded as an elite source of genes for crop improvement (Ellis et al., 2000). Recently, Tibetan wild barley is proved to be rich in genetic diversity of stress tolerance and barley quality. For instance, in comparison with cultivated barley, the wild barley shows greater variation in *HvGlb1*, encoding β-glucanase isoenzyme (Jin et al., 2011), β-amylase activity (BAA) and β-amylase thermostability (BAT) (Zhang et al., 2014). In the previous studies (Jin et al., 2011; Zhang et al., 2014), we found that XZ72 and XZ95 have higher activities of starch-degrading enzymes. So the two Tibetan wild barley accessions were used in this study.

In this study, a GC–MS-based strategy was used to investigate the impact of GA and ABA additions on metabolite profiles in the germinating seeds of the two Tibetan wild barley accessions during malting.

2. Materials and methods

2.1. Malting procedure

Two Tibetan wild barley accessions, i.e. XZ72 and XZ95 were malted in Joe White Malting System (Adelaide, SA, Australia). The malting procedures were as below: (1) Steeping stage: 5–8–8–1 2–4–5–2 h (wet–dry–wet–dry–wet–dry–wet); (2) Germination stage: 96 h at 16 °C. GA (0.5 ppm) or ABA (0.5 ppm) was added at the last wet stage during steeping by adding them into individual containers. Seed samples were collected at 24 h, 48 h, 72 h and 96 h after germination, and freeze dried, and then grinded for further analysis.

2.2. Determination of enzyme activity and β-glucan content

The activities of α-amylase, β-amylase, β-glucanase and limit dextrinase, and β-glucan content in the collected samples were measured using Megazyme assay kits (Megazyme International, Bray, Ireland), according to the manufacture's manual instruction.

2.3. Metabolite extraction

The grain samples collected at 0 h, 48 h and 96 h during malting were used for metabolic analysis. The metabolites were extracted according to Gorzolka et al. (2012). The pestled samples of 10 mg was homogenized using a ribolyzer (3 × 45 s, 6.5 m/s) and 1 ml of 80% methanol containing 10 μM ribitol as internal standard. Totally 750 μl of supernatant was dried in a vacuum freeze dryer and the dried sample was derivatized at 37 °C by adding 100 μl methoxyamine-hydrochloride (20 mg/ml in pyridine, Sigma–Aldrich) for 90 min and afterwards 100 μl MSTFA (Sigma–Aldrich) for 30 min.

2.4. GC–MS analysis

The extracted samples were determined for metabolites by 6890N GC/5975B MSD (Agilent, USA). The temperature-rising program was as follows: 80 °C for 3 min, 5 °C/min rate up to 300 °C, 2 min for 300 °C. Mass spectra of eluting compounds were identified using the commercial mass spectra library NIST (http://www.nist.gov) and the Golm Metabolome Database of the Max Planck Institute of Molecular Plant Physiology, Germany. (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd_msri.html).

2.5. Data analysis and statistics

Principle component analysis (PCA) of the identified metabolites was conducted using Simca-P v13.0 (http://www.umetrics.com/simca). Heatmap analysis was conducted using Excel 2007.

3. Results

3.1. The changes of degrading enzymes activities and β-glucan content as affected by GA and ABA treatments during germination

The changes of degrading enzyme activities and β-glucan content in the germinating grains of the two barley accessions were determined every day during germination. The activities of the enzymes, including α-amylase, β-glucanase and LD showed the dramatic increase, while β-amylase activity remained little change. Meanwhile, β-glucan content declined greatly during germination (Fig. 1a–e).

GA treatment markedly increased the activities of α-amylase, β-glucanase and LD in both barley accessions, but had little effect on β-amylase activity and β-glucan content (Fig. 1a–e). By contrast, ABA treatment caused a significant reduction in β-glucanase activity by 40–50% after 4 d germination (Fig. 1c). Interestingly, β-amylase activity in XZ95 was markedly reduced from 1 d to 3 d of germination, while XZ72 showed slight reduction after 3 d treatment (Fig. 1b). In addition, ABA significantly inhibited degradation of β-glucan in XZ72, but had relatively small effect on that in XZ95 (Fig. 1e).

Moreover, it is wealthy noted that XZ72 has constantly higher activities of α-amylase, β-glucanase and LD than XZ95 in both control and GA treatment after 2 d germination (Fig. 1a, c, and d). Although GA increased activities of all examined degrading enzymes, the difference between the two barley accessions was distinct, with XZ 95 having much lower activities of β-glucanase and LD than XZ72 (Fig. 1c and d). By contrast, XZ72 had lower β-amylase activity and β-glucan content in comparison with XZ95 (Fig. 1b and e).

3.2. The changes in metabolite profiles during germination and their response to GA and ABA treatments

Totally 52 metabolites with known structure, including 19 sugars, 15 organic acids, 15 amino acids and 3 other compounds, were identified. A principal component analysis (PCA) of the total metabolic data revealed the difference in metabolite profiles among samples (Fig. 2). The largest difference was found among the samples collected in different germinating time, as shown by the complete separations among metabolic samples in PCA, indicating that considerable metabolic changes happened during germination. The similar metabolite profiles could be found in XZ72 and XZ95 at the beginning of germination (0 d), and then the difference became larger with germination, suggesting the genotypic difference in metabolite change pattern or rate during germination. Clear separation among the treatments (CK, GA and ABA) was also detected in XZ72 after 2 d and 4 d germination, indicating that both GA and ABA treatments substantially affect germinating pattern. Moreover, the treatments had less effect on metabolites in XZ95, especially after 4 d germination, indicating the genotypic difference in the responses to two hormones during germination.

3.3. The responses of metabolites to GA and ABA treatments

According to Heatmap analysis, most of sugars or sugar derivatives, including sucrose, glucose, galactose, xylose, gentiobiose, trehalose, ribonic acid and xylitol were significantly increased, while raffinose and 2-keto-gluconic acid showed dramatic decrease during germination (Fig. 3). Meanwhile, most of amino acids increased, but glycine and alanine concentrations were reduced

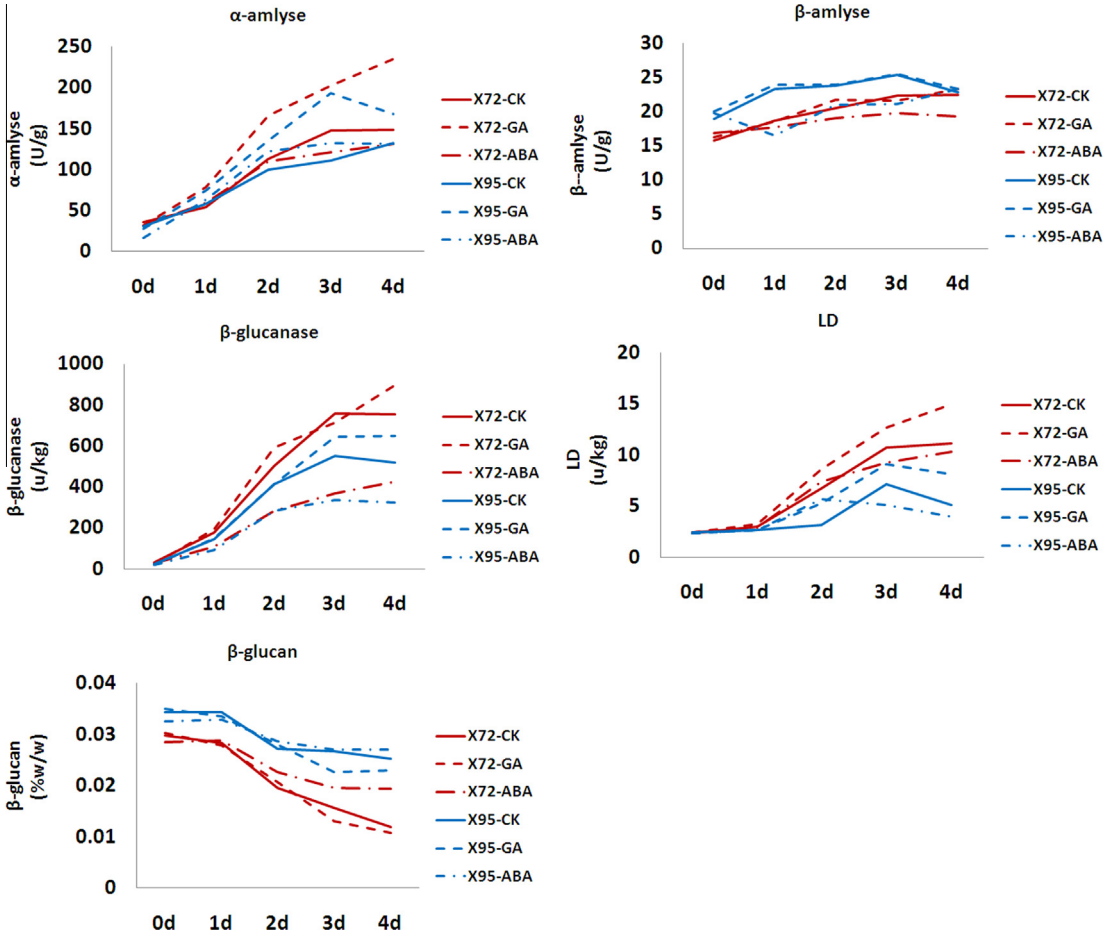

Fig. 1. Variation in the activities of α-amylase, β-amylase, β-glucanase and limit dextrinase, and β-glucan content in micro-malted barley.

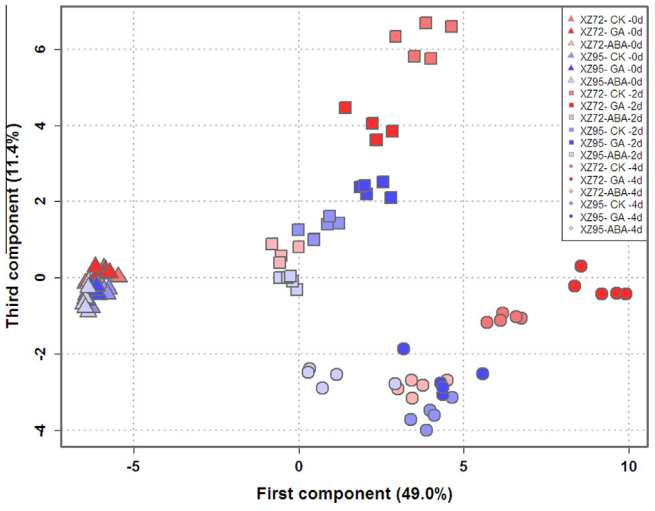

Fig. 2. Principle component analysis (PCA) of metabolite profiles.

during germination. A dramatic decrease was observed for some organic acids, such as pyruvic acid, malonic acid, fumaric acid and α-ketoglutaric acid, while some other organic acids showed increase, including citric acid, quinic acid, isocitric acid and 2-aminobutyric acid (Table 1).

At the initiation of seed germination, all samples showed the similar levels of metabolites. However, higher metabolite level could be detected in XZ72 than in XZ95 for both control and GA treatment after 4 d germination. XZ72 had significantly higher concentrations of most sugars and amino acids, including glucose, galactose, xylose, gentiobiose, trehalose, isomaltose, ribonic acid, erythritol, threonine, serine, valine, norleucine, pyroglutamic acid, proline, methionine, phenylalanine, ornithine and tryptophan, in GA treatment than in control after 4 d germination. However, XZ95 had little difference in most metabolites between control and GA treatment, although a slight increase could be found for some organic acids in the GA-treated samples. It may be suggested that GA treatment improved germination, as reflected by increased levels of sugars and amino acids, but the influence of GA treatment on germination is genotype-dependent. The finding is basically consistent with the results reached by PCA analysis. On the contrary, the concentrations of most metabolites were lower in ABA treatment than in control and GA treatment, indicating that ABA inhibits seed germination.

4. Discussion

In the current study, XZ72 and XZ95 showed similar trend in starch degrading enzymes activities and β-glucan content as affected by GA and ABA treatments (Fig. 1), but the differences between two cultivars became predominant during germination. The metabolites of XZ72 and XZ95 had the similar content at the very beginning of germination, and differed between the two genotypes when the germination entered the second day (2 d). XZ72 had significantly higher metabolite level than XZ95 at the 4 d of germination (Fig. 3). The enzymes activities showed the consistent changes as metabolites.

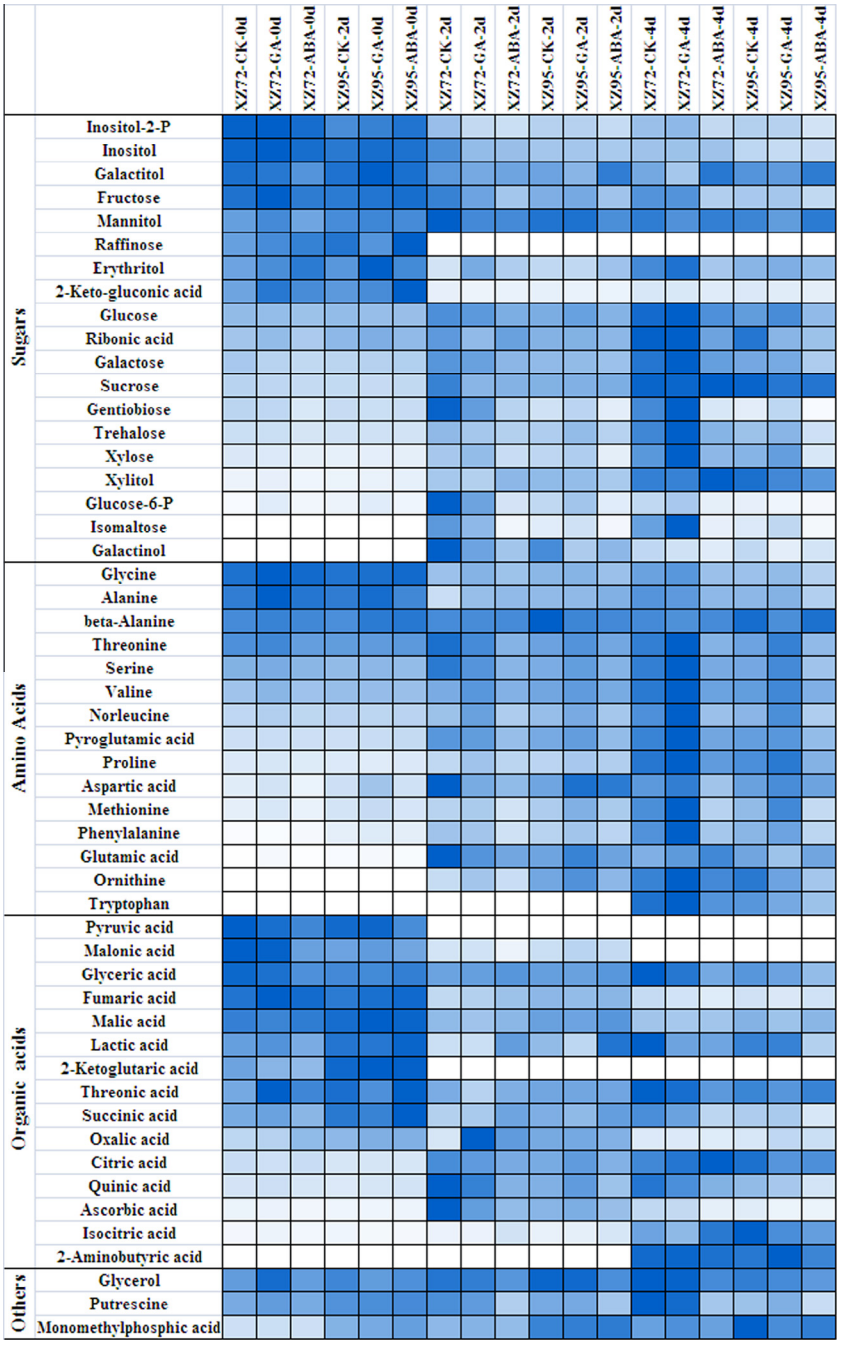

Fig. 3. Heatmap of metabolites at different stages of germination. The darker of the color means the higher abundance of the metabolites. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

We found that under the normal condition without addition of GA or ABA, most of the sugars, sugar derivates and amino acids increased during germination, being consistence with the observations in other metabolome studies on barley and rice (Frank et al., 2011; Gorzolka et al., 2012; Howell et al., 2008; Shu, Frank, Shu, & Engel, 2008). The increased levels of sugars and amino acids can be attributed to the raised activities of starch-degrading and proteolytical enzymes (Briggs, 1998; Evans, Li, & Eglinton, 2008; Evans, Li, Harasymow, Roumeliotis, & Eglinton, 2009), as reflected by the increased activities of α-amylase, β-amylase and LD (Fig. 1).

However, a few sugars showed the decrease, with raffinose having a dramatic decrease. Raffinose was known as a contributor of energy supply at the beginning of germination, and blocking of raffinose breakdown inhibits seed germination (Blochl, Peterbauer, & Richter, 2007). Fructose and inositol are two other metabolites in sugar group showing gradual decrease during germination (Fig. 3), and can react to build sucrose and galactinol, respectively (Gorzolka et al., 2012), thus explaining the reduction of inositol and raffinose, and increase of galactinol. However, different trend of fructose and inositol was observed in other metabolome study on germinating barley seeds, in which these metabolites showed increase at early stage of germination, reached a peak at mid-term stage, and declined gradually at late stage (Gorzolka et al., 2012). The inconsistency in these experiments may be attributed to the difference in malting procedure, such as soaking time, temperature, moisture and so on, but it need to be confirmed by further studies.

Malic acid and citric acid were the only two organic acids with high abundance in TCA cycle (Frank et al., 2011; Gorzolka et al.,

Table 1
The change of identified metabolites by GC–MS of different treatment compared with CKs were listed as below.

		X72-0 d		X72-2 d		X72-4 d		X95-0 d		X95-2 d		X95-4 d	
		GA/CK	ABA/CK	GA/CK	ABA/CK	GA/CK	ABA/CK	GA/CK	ABA/CK	GA/CK	ABA/CK	GA/CK	ABA/CK
Sugar	Erythritol	1.226	1.436	2.964	1.771	1.198	0.472	1.534	1.131	0.992	1.531	1.083	0.893
	Xylose	0.942	0.702	1.208	0.622	1.541	0.694	1.098	1.061	1.227	0.429	1.287	0.326
	Xylitol	1.243	0.888	0.855	1.299	0.985	1.253	1.097	1.217	0.957	0.779	0.829	0.726
	Ribonic acid	1.160	0.912	0.681	0.924	1.010	0.580	1.136	0.974	1.022	0.943	0.540	0.450
	2-Keto-gluconic acid	1.485	1.263	0.782	0.899	0.959	0.826	1.115	1.551	0.882	0.837	0.837	0.779
	Mannitol	1.220	0.940	0.738	0.735	0.842	0.984	1.047	1.007	1.016	0.806	0.812	1.085
	Galactitol	0.947	0.752	0.836	0.884	0.641	1.544	1.134	1.020	0.809	1.410	0.934	1.225
	Trehalose	1.005	0.791	0.802	0.677	1.330	0.631	1.015	0.936	1.347	0.904	1.213	0.505
	Inositol	1.044	0.952	0.596	0.570	1.004	0.998	1.084	1.057	1.008	0.947	0.878	0.850
	Glucose-6-P	3.522	1.650	0.573	0.172	1.413	0.430	1.628	0.869	1.479	0.502	0.675	0.488
	Inositol-2-P	1.015	0.931	0.588	0.506	1.127	0.601	1.092	1.217	1.005	0.788	0.971	0.588
	Gentiobiose	0.944	0.556	0.630	0.286	1.361	0.211	0.937	0.978	1.408	0.600	2.216	0.277
	Isomaltose	0.000	0.000	0.694	0.092	1.687	0.165	0.000	0.000	1.528	0.404	1.806	0.336
	Galactinol	0.000	0.000	0.579	0.368	0.766	0.502	0.000	0.000	0.455	0.628	0.468	0.713
	Fructose	1.136	0.936	0.739	0.460	0.996	0.452	1.046	1.097	1.062	0.738	1.045	0.703
	Glucose	0.973	0.898	0.914	0.735	1.081	0.751	0.962	0.948	1.144	0.926	1.162	0.699
	Galactose	0.818	0.703	0.890	0.692	1.164	0.704	1.089	1.139	0.981	0.896	0.984	0.596
	Sucrose	0.924	0.840	0.595	0.621	0.992	1.034	0.964	1.019	1.004	1.037	0.878	0.890
	Raffinose	1.209	1.328	0.000	0.000	0.000	0.000	0.772	1.154	0.000	0.000	0.000	0.000
Amino Acid	Alanine	1.226	1.055	1.939	2.031	0.953	0.664	1.114	0.910	1.017	1.147	1.068	0.669
	Valine	1.224	1.012	1.247	0.935	1.197	0.696	1.009	0.973	1.125	0.945	1.210	0.819
	Norleucine	1.201	1.008	1.525	0.838	1.439	0.567	0.955	1.003	1.285	0.867	1.518	0.686
	Glycine	1.124	1.050	1.231	0.977	1.115	0.670	1.028	1.073	1.041	0.863	1.096	0.739
	Serine	1.057	0.932	0.805	0.558	1.250	0.661	1.065	0.987	1.191	0.924	1.350	0.690
	Threonine	1.078	0.881	0.812	0.531	1.242	0.591	1.013	1.034	1.164	0.942	1.372	0.752
	Beta-alanine	1.066	1.026	0.996	1.008	0.945	0.990	1.172	1.199	0.762	0.740	0.770	0.973
	Methionine	1.449	0.823	1.184	0.634	1.418	0.406	1.306	0.937	1.543	1.021	1.730	0.546
	Pyroglutamate	1.065	1.000	0.950	0.628	1.291	0.723	1.086	1.177	1.152	0.794	1.076	0.690
	Aspartate	1.503	0.706	0.530	0.437	1.261	0.580	1.843	0.941	1.574	1.460	1.214	0.972
	Phenylalanine	0.963	1.622	0.964	0.504	1.460	0.520	1.281	1.056	1.315	0.962	1.261	0.580
	Glutamate	5.888	2.051	0.674	0.559	1.268	1.486	1.232	0.906	1.367	1.005	0.674	1.000
	Ornithine	0.000	0.000	1.627	0.962	1.285	0.953	0.000	0.000	1.242	0.819	0.687	0.429
	Tryptophan	0.000	0.000	0.000	0.000	1.136	0.764	0.000	0.000	0.000	0.000	0.817	0.584
	Proline	1.085	0.920	1.510	1.073	1.176	0.741	0.888	1.016	1.265	1.134	1.177	0.687
Organic acid	Pyruvic acid	0.905	0.749	0.000	0.000	0.000	0.000	1.019	0.770	0.000	0.000	0.000	0.000
	Lactic acid	1.126	0.873	0.992	2.904	0.573	0.565	0.986	1.114	0.614	2.038	1.000	0.375
	Oxalic acid	1.102	1.679	6.220	3.897	1.022	0.937	1.124	1.118	1.020	0.921	1.611	1.315
	2-Aminobutyric acid	0.000	0.000	0.000	0.000	1.023	0.954	0.000	0.000	0.000	0.000	1.177	0.900
	Malonic acid	0.982	0.588	1.062	0.449	0.000	0.000	1.088	0.969	1.314	1.116	0.000	0.000
	Succinic acid	1.107	0.867	1.122	1.927	0.823	0.373	0.926	1.189	0.869	1.190	1.047	0.476
	Glyceric acid	0.939	0.734	1.043	1.137	0.855	0.543	0.998	1.086	0.977	1.089	0.887	0.647
	Fumaric acid	1.147	1.063	1.225	1.598	0.790	0.567	1.085	1.133	0.930	1.019	0.807	0.987
	Malic acid	0.966	1.026	0.859	1.042	0.936	0.988	1.091	1.052	0.978	1.118	0.785	0.950
	Threonic acid	1.841	1.389	0.550	0.962	0.912	0.644	0.775	1.103	1.000	1.005	0.854	1.011
	2-Ketoglutaric acid	0.815	0.755	0.000	0.000	0.000	0.000	1.062	1.050	0.000	0.000	0.000	0.000
	Citric acid	0.911	0.990	0.883	0.736	1.146	1.337	1.035	1.025	1.191	0.929	0.747	0.784
	Isocitric acid	1.504	1.098	1.000	2.256	0.769	1.455	0.719	0.675	1.123	1.720	0.713	0.608
	Ascorbic acid	1.036	0.740	0.618	0.424	0.952	0.411	0.967	1.028	1.091	0.859	0.668	0.667
	Quinic acid	1.198	0.939	0.793	0.485	0.825	0.575	1.142	1.246	1.262	0.767	0.813	0.405
Others	Monomethylphosphate	1.012	1.001	1.069	0.940	1.174	0.997	1.098	1.236	1.041	1.062	0.714	0.810
	Glycerol	1.483	0.984	0.945	0.771	0.977	0.726	0.823	0.923	0.979	0.738	0.903	0.788
	Putrescine	1.173	0.995	0.893	0.437	0.928	0.351	1.027	1.057	1.011	0.669	1.301	0.569

2012). The current study showed that citric acid level increased while malic acid level decrease in the germinating barley seeds, being consistent with other observations (Frank et al., 2011; Gorzolka et al., 2012; South, 1996). In addition, putrescine increased at late stage of germination, especially in XZ72 under control and GA treatment. The similar results were also observed by Izquierdo-Pulido, Mariné-Font, and Vidal Carou (1994). Putrescine is usually formed from ornithine due to endogenous decarboxylase activity and plays a vital role in plant metabolism (Halász, Baráth, & Holzapfel, 1999).

In this study, addition of GA increased activities of α-amylase and LD in both barley accessions, which is in accordance with previous observations (Enari & Sopanon, 1986; Filner & Varner, 1967; Hardie, 1975; Schroeder & MacGregor, 1998). The enhanced enzyme activity may partially explain the elevated levels of sugars and sugar derivates, especially in GA-treated XZ72. However, sugars were little or slightly affected by addition of GA in XZ95. The possible explanation might be genotypic difference in endogenous GA level or response to heterogeneous GA. Actually the genotypic difference in response to GA was once reported (Evans et al., 2009). The lower LD activity in XZ95 relative to XZ72, may partially explain its lower sugar level. It is not surprising that β-amylase in germinating seeds has no response to GA, as the enzyme is synthesized during grain development (Hardie, 1975).

Amino acids showed increase in the GA-treated seeds, especially for XZ72 (Fig. 3). Obviously it may be attributed to enhanced protease activity by GA, as it was reported by some researchers (Briggs, 2002; Enari & Sopanon, 1986). The released amino acids could provide the sufficient substrates for biosynthesis of proteins and enzymes, being necessary for seed germination. GA also promotes cell wall modification and degradation (Briggs, 2002; Enari & Sopanon, 1986), as reflected by increased β-glucanase activity

(Fig. 1), a key enzyme involved in cell wall degradation (Forrest & Wainwright, 1977). Higher level of galactose, which is a product of cell wall degradation in GA treatment than in CK, may also support the finding (Fig. 3).

Seed germination has been proved to be regulated by antagonistic interaction of ABA and GA, whereby GA promotes and ABA inhibits germination (Bewley, 1997; Holdsworth, Bentsink, & Soppe, 2008). It is well documented that addition of GA at steeping may improve malt quality through enhancing some sugar-degrading enzymes (Briggs, 1998), while ABA has an antagonistic effect to GA (Bewley, 1997). The current study confirms the substantial effect of GA and ABA on the metabolite profiles of germinating barley seeds, and may make further insight into the role of these hormones in seed germination.

5. Conclusions

In this study, we used GC–MS to analyze changes of metabolites for two Tibetan wild barley accessions XZ72 and XZ95 during germination and as affected by addition of GA and ABA. α-amylase, β-glucanase and LD increased dramatically, and β-amylase activity remained little change during germination. Meanwhile, β-glucan content declined greatly. GA treatment caused great increase in the activities of some sugar-degrading enzymes, and ABA had the opposite effect. Sugars and amino acids are most changed compounds, and GA or ABA treatment enhanced or inhibited the accumulation of some metabolites, respectively.

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