Direct Infusion Metabolomics of a Drought Tolerant Plant Introduction Soybean Cultivar

Introduction

Agricultural crops endure a matrix of stress resultant from a variety of sources including biota or abiotic stressors such as drought, flooding, salinity, or nutrient availability.[1] Albeit among the different sources plants can endure, water deficiency is the most prolific and detrimental to farms. Soybean (*Glycine max)* has a particular intolerance to water deficiency in the early stages of growth and flowering, where a 50% decrease in water availability can result in up to a loss of half the expected yields. This decrease in productivity subsequently results from a matrix of phenotypic expressions and secondary metabolic responses ensuing in decreased rates of growth and productivity.[2] Although water deficiencies do not directly impact the photosystem, the secondary impacts triggered from stomatal closures and in severe cases clamping of leaves, decrease carbon dioxide fixation and increase flux of both primary, and secondary metabolites.[3] This progressive decay in the photosynthetic productivity of the plant and relative phytochemical content can thereby be utilized as a metabolomic indicator of the breadth of the photosynthetic antenna, and a marker of the health and productivity of the plant. This targeted approach of profiling the photosystem has been demonstrated as such to be used in studies approaching abiotic stressors, such as drought and physiological processes such as senescence.[4-5]

Prior greenhouse and field trials have demonstrated endogenous stress tolerances from plant introductions (PI) of exotic cultivar into new terroir, *PI 536731* was grown in field trials alongside of *Pana*.

Due to the high levels of variability of metabolites through various stages of growth and stress responses, one all-inclusive method for simultaneous detection of all primary and secondary metabolites is not available. Researchers use multiple platforms, including gas or liquid chromatography (GC/LC) coupled with mass spectrometry (MS), nuclear magnetic resonance (NMR), and a variety of spectroscopic techniques including targeted fluorescence for the study of chlorophyll (Chl), pheophytin (Pheo). However due to large cohorts required for these analysis with hundreds of differential effects existing from singular stressors, these methods prove to be low-throughput. High-resolution MS platforms such as Fourier Transform ion cyclotron resonance (FT-ICR) due to unprecedented mass resolving power and mass accuracy allows for the direct infusion of samples with no on-line separations[6], allowing for at least a ten-fold decrease in analysis time and acquisition of hundreds of metabolic signals. Herein described is the target profiling of primary metabolites in the photosystem and related metabolites of a drought tolerant cultivar of Soybean utilizing direct infusion FT-ICR MS.

Methods

Materials

Quinapril HCl used was a USP Reference Standard. Methanol (HPLC Grade) was from Sigma-Aldrich. Formic Acid 88% (Certified ACS) was from Fisher Scientific.

Samples Used During the Study

Two cultivar of soybean (*Glycine max) PI 536731* and *Pana* were grown in field trials at the University of Missouri (latitude 38.895305, longitude -92.205917). Drought treatment consisted of no irrigation and rainfall in the field for 3 weeks prior to collection and control conditions were within irrigated plots, two days prior to collection the plots were irrigated. Plants at the time of collection were collected in two physiological age groupings, young at 1 week and old at 2 to 3 weeks. After collection leaves were flash frozen at -80˚C and stored in polycarbonate petri-dishes at -20°C until extractions were processed.

Extraction Protocol

The experimental and control groups underwent a pooling of plant tissue from the various leaves collected, the samples were flash frozen by liquid nitrogen and macerated in an aliquot of solvent with an internal standard added. Maceration continued for five minutes and particulate matter was subsequently removed through vacuum filtration. Samples were dried in a vacuum oven at ambient temperatures and diluted to constant volume.

Data Collection and Processing

All spectra were acquired on a Bruker Daltonics 12T SolariX FT-ICR mass spectrometer by direct infusion electrospray ionization of the samples. Datasets were collected using broadband detection with 100 scans, and a transient of 0.4436 seconds. Collision induced dissociation (CID) was used to confirm the identity of chlorophyll-related metabolites. No charging additives were added due to sufficient signal in positive ionization mode, formic acid was added to reduce adduction during CID experiments.

Solvent extracts were analyzed in triplicate instrumental and technical replicates of the two cultivars for for young (1 week) and old (2-3 weeks) leaves under normal and under drought conditions. Spectra were processed in Bruker DataAnalysis 5.0 with a signal-to-noise ratio of 5 for peak picking and exported from DataAnalysis into the online web platform MetaboAnalyst 4.0 for statistical analysis, and METLIN was used for metabolite annotation. Filtering based upon standard deviation was used to remove irreproducible data points and normalization was completed to the peak area of the internal standard to remove variance from direct infusion. Log fold changes were generated through transformation of the data.

Results and Discussion

Multivariate Analysis of Cultivar Datasets

Principle component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed in order to discriminate subsets of drought treatment in the cultivar in the MetaboAnalyst 4.0 package, shown in Figure 1 are the three dimensional models of the old populations. The models demonstrate the distinctive separations of the control and drought treated metabolic fingerprint, also the distinction between the test and check cultivar of soybean.

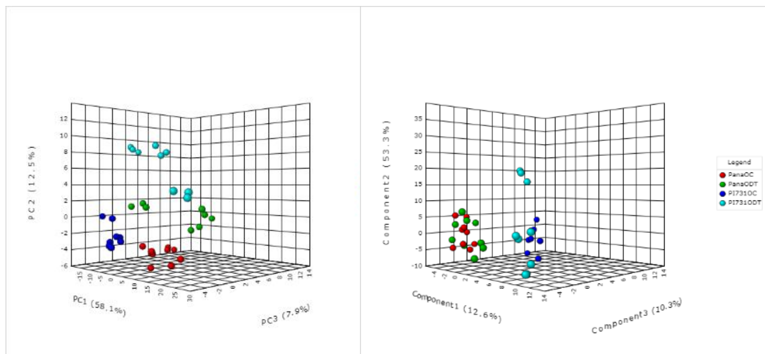

Figure 1. Three dimensional scores plot of a PCA (left) and PLS-DA (right) of old control (OC) and drought treated (ODT) Pana and PI 567731

Alongside of the drought treatment, the period of two weeks of growth was a distinguishable influence, as shown in Figure 2. Within this model, the use of confidence intervals surrounding each subset allows for linkage of the scales of the components to that of the association of the treatment. From control to treatment in the case of *PI 567731* it is observed that within the metabolic fingerprint both young and old drought treatments have molecular compositions closer to that of the control than the check cultivar. As to not impart effects of growth on the multivariate analysis, the control and experimental treatments were analyzed independent of age.

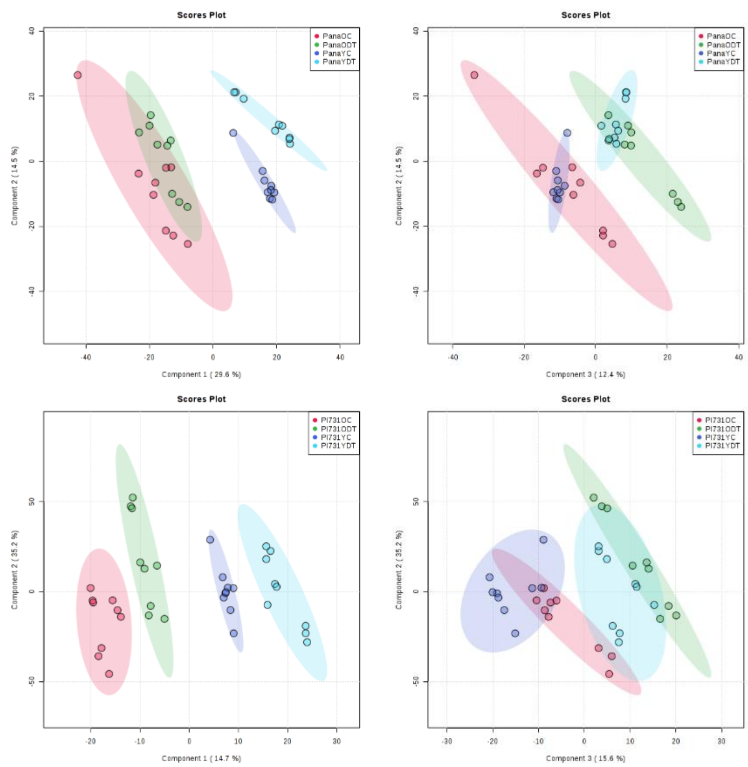

Figure 2. PLS-DA score plots of the first and second components (left) and the second and third components (right) of all subsets of Pana (top) and PI 567731 (bottom) samples. With a window of 95% confidence around the encompassed variance.

Enhanced Levels of Chlorophyll and Related Metabolites in Drought Treatment

Increased levels of Chl a and b, and Pheo a were observed within the old and young samples of the plant introduction cultivar, with an overall median increase in all concentrations of metabolites detected within the *PI 536731* cultivar. The relative abundances and ratio of these metabolites have been previously demonstrated as secondary links to the holistic health of the plant, as demonstrated in previous works on Chl and related primary metabolites as markers for stress tolerance.[7] Where the increased concentrations of Chl b can be a signet of productivity between cultivar. On average the observable signal resulted in an enhancement of an average of one log-fold change detected for *PI 567731*; these relative levels were maintained in the plant introduction under the drought treatment whereas *Pana* had decreased changes in median concentrations, which also is observed for Pheo a. On top of a general increase, the enhanced expression of Chl b, and Pheo a marks

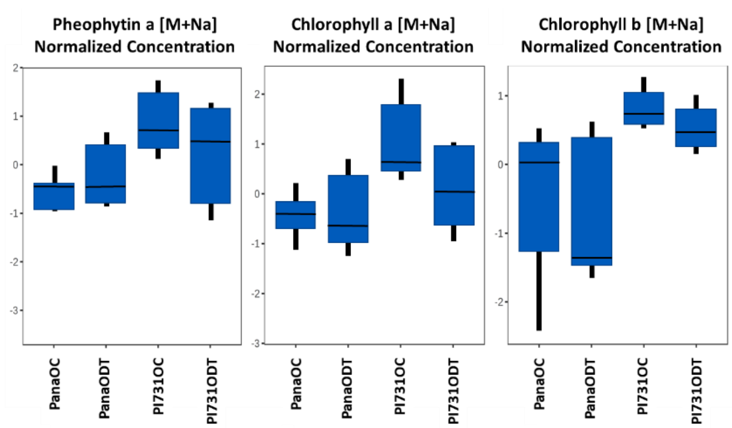

Figure 3. Log scale box and whisker plots of Chl a, Chl b, and Pheo a show relative concentrations between old control (OC) and old drought treated (ODT) samples of Pana and PI 567731 samples.

proliferation of the photosystem, increasing the breadth of the photosynthetic antennae in plants with positive stress responses.[8] Within studies upon maize and rice, with known water deficient intolerances, the photosynthetic antenna has been shown to be broadened and an altered ratio of Chl a/b within tolerant cultivar. These metabolic pathways otherwise should experience a downregulation after the phenotypic and physiological responses, which was not observed relative to the irrigated soybean plants, reinforcing drought tolerance with respect to a targeted approach at profiling the Chl metabolism.

Tandem Mass Spectrometry of Novel Chlorophyll Related Metabolites

Within the metabolites annotated was a distinct novel chlorophyll related metabolites previously reported within soybean extracts by Yilmaz et al.[5] Through the fragmentation of the molecules by collision induced dissociation (CID), these species (>893 Da) show characteristic losses to that of a porphyrin base with unidentified moieties attached directly to the porphyrin with no losses or changes to the phytyl group; an example is indicated in Figure 4. Of particular interest is the sodium adduct of a chlorophyll related metabolite at 1073.705 Da, which has a significant increase in concentration, and decrease in variability in the *PI 536731* subsets. The median increase in relative concentration is a five-fold enhancement in comparison to *Pana,* shown in the box and whisker plot in Figure 5. Once isolated a product ion at 893.55473 Da forms, corresponding to a sodium adduct of Pheo a through the neutral loss of 180.15031 Da. When searched against METLIN and LIPID MAPS this yields matches with a conjugated trenoic fatty acid (C__H__O). Upon further fragmentation neutral losses of 278.29692 Da and 76.01606 Da appear. Corresponding to the loss of the phytyl group (C20H38) to pheophorbide a, and further loss of moieties on the porphyrin ring (C2H5O3) respectively. Consistent with previously reported literature studies of fragmentation of both Chl a[11] and Pheo a.[12]

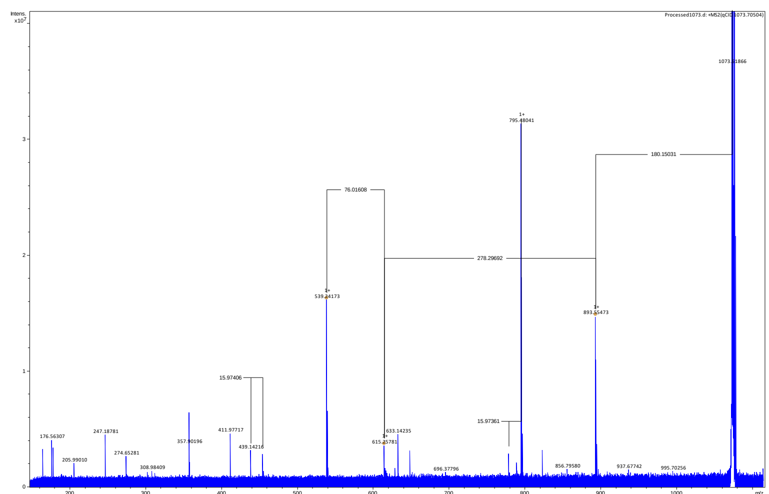

Figure 4. CID tandem mass spectrum of a chlorophyll-related metabolite (CRM) with a mass of 1073.7 Da. The fragments prove that this previously unidentified substance possesses the core porphyrin structure of chlorophyll.

The generation of this neutral loss under weak collisional energies and the loss of an intact phytyl suggests a weak bond formed to the porphyrin ring, not a modification that is not directly linked to the group. Literature reports the membranes of chloroplasts to have increased levels of conjugated free fatty acids (FFAs), such as hexadecatrenoic acid. These FFAs are postulated to be pertinent to regulation of chloroplasts especially under low or high temperatures, and abiotic stress.[9-10] The addition of a trenoic acid to the porphyrin ring, alongside the large fold increase in concentration within the drought tolerant plant introduction cultivar suggests this related metabolite as portion of a widened photosynthetic antenna, as for increased levels of Pheo a and Chl b. However, the metabolic pathway for the attachment and position on the porphyrin ring is still unknown, fragmentation confirms relation into the porphyrin metabolism for these chlorophyll related metabolites.

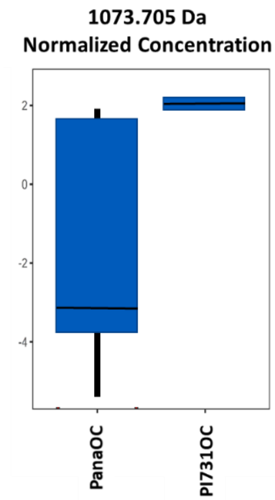

Figure 5. Log scale box and whisker plots of a sodium adduct of Chl related metabolite 1073.705 between Pana and PI 536731 old control (OC) samples.

Library of Metabolites for PI536731

Because the cultivar PI536731 is a drought-tolerant plant, our studies were particularly interested in obtaining a library of molecular components from leaf extracts of these plants under normal conditions and under drought-stress conditions. In construction of the library, we utilized two separate ages for the leaf metabolites, a young group (1 week of sprouted leaves) and an old group (2-3 weeks of sprouted leaves). Controls were grown under normal irrigation conditions. In addition, these same two groups were also grown under drought stressed conditions as described in the Experimental Section. Table 1 presents a library of m/z values for the molecular components from these four groups (labeled Old Control, Old Drought, Young Control, Young Drought). To qualify for inclusion in the table, an ion had to be detected in each of three technical replicates from three individual leaves (thus, detected n = 9 times). The Young Control group has the most metabolites, at 241, while the Young Drought group has 171. The Old Control group has 197 metabolites, while the Old Drought group has slightly more, at 205. A library is also being constructed for the Pana group. As noted above, many of these metabolites have been identified as secondary metabolites of chlorophyll; while their full identities are not known, they do not correlate with any currently known chlorophyll metabolites. Therefore these are previously unreported compounds indigenous to soybean leaves. Once the library from Pana is fully constructed and more of the metabolites have been identified, we will embark on a systems biology networking analysis to determine how the metabolites are related to one another. It is possible there are multiple metabolic pathways occurring in the soybean leaf, and that because of its drought-tolerance, PI536731 may be able to adapt to drought conditions in ways that Pana cannot.

Acknowledgements

We gratefully acknowledge Dr. Henry Nguyen and Dr. Heng Fang at the University at Missouri for providing the samples of the two soybean cultivars presented in this study. We are grateful for the support of the New York Corn and Soybean Growers Association for the financial support for this research, the National Institutes of Health (NIH) for support in obtaining the FT-ICR mass spectrometer, and the Chemistry Instrument Center at the University at Buffalo for housing the mass spectrometer.

References

1. Pandey, P.; Irulappan, V.; Bagavathiannan, M. V.; Senthil-Kumar, M., Impact of Combined Abiotic and Biotic Stresses on Plant Growth and Avenues for Crop Improvement by Exploiting Physio-morphological Traits. *Frontiers in Plant Science* **2017,** *8* (537).
2. Mutava, R. N.; Prince, S. J. K.; Syed, N. H.; Song, L.; Valliyodan, B.; Chen, W.; Nguyen, H. T., Understanding abiotic stress tolerance mechanisms in soybean: A comparative evaluation of soybean response to drought and flooding stress. *Plant Physiology and Biochemistry* **2015,** *86*, 109-120.
3. Pinheiro, C.; Chaves, M. M., Photosynthesis and drought: can we make metabolic connections from available data? *Journal of Experimental Botany* **2010,** *62* (3), 869-882.
4. Iqbal, N.; Hussain, S.; Raza, M. A.; Yang, C.-Q.; Safdar, M. E.; Brestic, M.; Aziz, A.; Hayyat, M. S.; Asghar, M. A.; Wang, X. C.; Zhang, J.; Yang, W.; Liu, J., Drought Tolerance of Soybean (Glycine max L. Merr.) by Improved Photosynthetic Characteristics and an Efficient Antioxidant Enzyme Activities Under a Split-Root System. *Front Physiol* **2019,** *10*, 786-786.
5. Yilmaz, A.; Rudolph, H. L.; Hurst, J. J.; Wood, T. D., High-Throughput Metabolic Profiling of Soybean Leaves by Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. *Analytical Chemistry* **2016,** *88* (2), 1188-1194.
6. Kirwan, J. A.; Weber, R. J. M.; Broadhurst, D. I.; Viant, M. R., Direct infusion mass spectrometry metabolomics dataset: a benchmark for data processing and quality control. *Scientific Data* **2014,** *1* (1), 140012.
7. Dalal, V. K.; Tripathy, B. C., Water-stress induced downsizing of light-harvesting antenna complex protects developing rice seedlings from photo-oxidative damage. *Scientific Reports* **2018,** *8* (1), 5955.
8. Kume, A.; Akitsu, T.; Nasahara, K. N., Why is chlorophyll b only used in light-harvesting systems? *Journal of Plant Research* **2018,** *131* (6), 961-972.
9. Routaboul, J. M.; Fischer, S. F.; Browse, J., Trienoic fatty acids are required to maintain chloroplast function at low temperatures. *Plant Physiol* **2000,** *124* (4), 1697-1705.
10. Yaeno, T.; Matsuda, O.; Iba, K., Role of chloroplast trienoic fatty acids in plant disease defense responses. *The Plant Journal* **2004,** *40* (6), 931-941.
11. Wei, J.; Li, H.; Barrow, M. P.; O'Connor, P. B., Structural characterization of chlorophyll-a by high resolution tandem mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2013**, *24(5)*, 753-760.
12. Wei, J.; O'Connor, P. B., Extensive fragmentation of pheophytin a by infrared multiphoton dissociation tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2015**, *29(24)*, 2411-2418.

Table 1. Library of mass-to-charge ratio (m/z) values for Molecular Components of PI536731 Cultivars under Normal Irrigation and under Drought-Stress. An (X) indicates this component was detected in the given group.

Old Control (OC)	Old Drought (OD)	Young Control (YC)	Young Drought (YD)
203.0512	217.068	203.0523	217.068
217.0682	219.027	217.0683	266.055
233.0423	293.064	233.0422	293.961
277.0895	301.149	293.0634	301.694
293.9609	306.123	301.1409	306.123
301.3568	308.120	317.1144	317.115
315.8932	317.115	331.1899	317.209
317.2089	317.209	333.1828	322.097
329.0331	322.097	333.2042	324.094
331.1881	324.094	335.0952	331.188
333.1828	329.053	335.2008	333.185
333.2038	333.183	335.427	333.204
335.0951	333.205	337.0896	335.983
337.0897	335.095	347.162	336.824
345.0067	337.090	349.1781	347.162
347.162	345.007	349.1985	349.178
349.1778	349.178	351.069	349.199
349.1985	351.069	351.1933	351.069
351.0689	351.215	351.2144	351.193
351.1936	364.321	353.23	351.215
351.2152	365.105	353.2665	353.230
364.3211	367.189	365.1727	365.398
365.1054	375.251	367.4098	367.188
365.1726	379.121	369.2039	367.209
367.1888	381.079	369.2404	369.204
367.2093	381.298	373.1778	369.241
375.2297	391.225	375.6847	389.033
375.2517	397.132	381.0795	391.225
381.0794	398.118	388.3831	397.132
383.1757	409.184	391.1659	397.272
391.2274	411.147	391.2247	398.127
395.0952	413.267	393.2404	411.147
397.1317	414.090	395.0952	413.267
407.1404	425.157	395.1737	414.090
409.1624	427.137	397.1319	429.240
409.1835	427.173	397.2717	435.251
411.1508	429.241	407.1401	439.142
411.1991	439.146	407.2198	440.221

413.2674	440.221	409.1835	447.163
414.0905	441.2975	411.1473	455.116
425.1573	445.391	413.2666	455.132
427.1366	447.581	414.0905	457.272
427.1731	449.375	425.1573	462.203
429.2407	455.116	427.1367	465.349
440.2218	455.313	427.1733	471.090
441.2978	457.271	429.2409	471.287
443.2917	459.168	439.1426	480.655
447.1633	462.203	440.325	483.075
449.3545	463.303	441.2981	485.302
449.3755	463.334	447.1672	485.341
455.1162	465.349	449.3756	487.064
457.7276	465.370	455.1317	503.334
463.3337	469.365	457.2718	518.468
465.3493	471.090	459.1682	523.303
469.3653	471.287	462.2035	547.371
471.0899	478.177	463.3338	557.093
471.2875	479.241	465.3494	559.357
478.1774	479.277	465.3705	567.329
479.2407	483.075	469.3653	575.491
481.3438	485.302	471.0699	577.371
483.0752	485.729	471.0904	587.549
485.3028	487.064	471.2876	591.393
485.3392	523.113	478.1774	595.485
487.0638	523.303	483.5779	603.522
487.1942	527.158	485.3033	607.391
501.3331	536.438	485.3393	607.470
511.3548	543.184	487.064	611.355
536.4377	547.361	487.1944	613.480
543.184	553.362	497.3404	615.465
553.2875	557.093	501.3342	615.552
559.1577	559.158	513.1372	617.148
579.4172	559.517	518.4683	629.454
583.6334	567.329	523.3037	631.470
587.5487	575.491	537.407	631.554
591.4173	583.415	559.1981	633.142
599.4162	584.275	567.5456	635.413
601.4744	587.549	569.3638	635.465
603.5225	589.257	571.4123	637.480
605.4554	591.417	573.5012	639.381
607.6088	593.276	575.6359	643.527
613.4804	595.487	577.1911	645.449
615.4971	599.386	587.5489	647.465

617.1478	599.411	599.3855	649.151
627.4386	605.240	601.4748	651.453
629.4542	607.391	601.5325	653.454
631.4709	611.355	603.5226	655.411
633.219	613.480	605.4553	655.920
635.4659	615.497	607.3916	657.688
639.3813	615.580	611.3556	659.308
639.496	617.148	613.3867	659.502
645.4518	629.454	613.4805	661.444
649.1166	633.858	615.4023	663.468
651.4398	635.002	615.4967	667.433
655.4698	639.381	617.1666	669.453
657.4855	639.692	617.4687	673.483
659.2872	641.512	629.4542	679.445
659.5017	641.526	631.47	685.224
661.6684	645.633	632.9489	687.236
667.436	649.117	633.1405	689.605
667.4544	651.441	633.4493	691.491
669.5371	653.524	635.4136	692.562
673.4823	655.382	635.4647	699.408
675.2612	655.468	637.4803	703.580
683.4306	657.485	639.3808	704.618
685.5315	659.287	639.4963	720.593
697.5906	659.502	641.5113	723.465
699.4221	667.437	643.5276	725.566
703.5816	667.454	645.4491	727.581
711.5543	669.539	645.4703	731.610
713.5633	679.445	647.466	736.534
713.5842	683.432	649.1169	743.434
715.5796	687.605	651.4388	743.575
719.5744	697.589	653.4568	748.623
721.6258	699.408	655.3821	752.508
725.5632	699.605	657.0378	787.460
727.5425	703.586	657.4973	787.579
727.5777	704.618	659.5024	791.917
729.5603	709.589	661.4532	797.518
735.106	711.548	663.4601	803.575
737.5999	711.605	667.4335	805.663
741.5678	713.563	669.4501	811.518
743.5379	721.626	669.5374	813.492
743.6471	725.563	673.4811	829.708
745.5557	727.577	675.2614	831.508
752.5079	729.564	677.4382	851.616
753.5943	731.283	679.4562	853.643

757.5549	736.534	685.4442	867.572
759.392	737.600	687.4601	869.702
767.6099	741.927	689.4759	871.574
771.6054	743.434	691.4911	873.600
775.0961	745.555	692.5601	873.694
779.2004	752.508	697.5918	875.513
783.6053	753.594	699.4075	875.618
787.5789	759.123	701.4393	875.712
787.6002	771.605	703.5793	882.643
789.6885	775.097	704.6181	891.687
791.5068	785.584	711.5486	895.678
795.1746	787.579	713.5635	897.695
795.6414	787.600	715.5798	901.646
797.5177	789.133	720.6893	905.663
803.5746	789.688	721.6261	907.680
803.6461	791.507	725.5642	913.672
805.678	795.641	727.5431	915.525
811.6366	797.518	729.5604	921.656
819.5708	801.572	731.2843	923.676
819.6414	803.574	731.6101	925.524
821.6573	805.177	735.5687	927.648
829.4857	805.662	737.6002	929.666
837.652	813.492	739.5793	931.497
845.4814	815.489	741.5613	937.586
868.6825	819.571	743.4347	937.654
870.77	821.127	743.5383	939.671
871.5735	821.657	743.6395	943.643
873.6953	829.486	745.3446	945.478
873.7252	835.748	747.0866	945.662
875.6167	837.652	752.5087	953.578
885.6726	853.642	753.5948	955.543
889.6684	857.867	755.5756	959.637
893.7756	859.846	757.5562	961.656
895.6783	871.589	761.5515	974.672
897.6953	873.694	769.5896	975.543
901.6471	875.617	771.606	975.632
905.6651	875.712	784.7341	977.652
909.5293	877.663	787.5794	981.591
911.5251	887.568	789.6892	996.642
911.6549	889.668	791.5083	1051.723
915.5242	891.685	795.6418	1057.732
925.5235	893.555	797.5185	1068.545
927.6489	895.679	803.5748	1073.706
929.5029	897.695	803.6454	1079.753

931.4979	901.647	805.663	1089.701
937.5856	901.726	811.636	
939.5476	903.744	813.7138	
943.6433	905.664	819.5718	
945.4805	909.529	819.642	
953.5597	911.653	821.152	
955.5439	913.878	821.6581	
959.5701	915.746	829.4854	
959.6395	925.540	835.5675	
965.6169	927.647	837.1257	
974.6726	930.200	837.6522	
975.5437	931.517	843.6861	
981.5902	937.585	851.6165	
991.4817	939.548	853.643	
996.6417	943.642	854.6126	
1035.747	945.480	857.6581	
1051.722	945.661	870.7706	
1057.732	953.560	871.5738	
1063.779	955.544	873.6529	
1067.716	959.570	873.7256	
1068.546	961.656	875.6202	
1073.707	965.617	875.7141	
1079.754	974.672	882.6442	
1089.698	975.544	889.6688	
1105.639	978.778	891.687	
1105.683	981.590	893.5561	
1121.69	985.552	897.6964	
	991.482	901.6481	
	996.642	905.6644	
	1035.747	907.6813	
	1051.722	909.5299	
	1057.732	911.6532	
	1063.779	913.6695	
	1073.707	915.5258	
	1105.694	921.6582	
		923.6761	
		925.5246	
		927.6483	
		929.6701	
		931.4979	
		937.5868	
		937.6536	
		939.5483	
		939.6714	

		943.6433 945.4781 945.6605 953.5898 955.5446 959.5705 959.9286 961.6565 965.6177 974.6734 975.5453 975.6325 976.7612 977.6531 981.5928 996.6427 1035.747 1051.723 1057.733 1067.715 1068.545 1073.706 1079.753 1083.505 1089.699 1105.698 1121.691	