|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Table S1 statistical table of sample sequencing data evaluation** | | | | | | |
|  | HX11 | HX22 | HX33 | ZS11 | ZS22 | ZS33 |
| Total Reads Count(#) | 20,460,235 | 21,840,510 | 23,786,119 | 24,605,718 | 22,067,134 | 28,832,952 |
| Total Bases Count(bp) | 6,095,092,806 | 6,528,806,510 | 7,096,527,656 | 7,350,637,532 | 6,595,931,570 | 8,601,828,626 |
| Q30 Bases Ratio(%) | 93.28% | 93.28% | 93.96% | 94.08% | 94.56% | 94.07% |
| GC Bases Ratio(%) | 42.15% | 41.94% | 42.05% | 41.70% | 42.13% | 41.98% |

|  |  |  |  |
| --- | --- | --- | --- |
| **Table S2 Annotation statistics of differential genes** | | | |
| #Anno\_Database | Annotated\_Number | 300<=length<1000 | length>=1000 |
| COG\_Annotation | 9742 | 3627 | 6115 |
| GO\_Annotation | 24533 | 11790 | 12743 |
| KEGG\_Annotation | 12032 | 5755 | 6277 |
| KOG\_Annotation | 18107 | 8510 | 9597 |
| Pfam\_Annotation | 22388 | 9308 | 13080 |
| Swissprot\_Annotation | 21189 | 9131 | 12058 |
| eggNOG\_Annotation | 29936 | 14628 | 15308 |
| nr\_Annotation | 34964 | 18310 | 16654 |
| All\_Annotated | 35805 | 19098 | 16707 |

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| **Table S3 Statistics of gene specific primers of selected DEG** | | | |
| Gene name | Primer name | Sequence(5'to3') | TM |
| EF-1α | EF-1α-F | GCACGCTCTTCTTGCATTTACT | 60 |
|  | EF-1α-R | GGGCTTGTCTGTGGGTCTCTT |  |
| primary amine oxidase activity | c76636.graph\_c1-F | TTTGATAGACTGGAGGGAGCAT | 60 |
|  | c76636.graph\_c1-R | CCCAGATAGCTCAATTATCTTCCG |  |
| shikimate O-hydroxycinnamoyltransferase activity | c64912.graph\_c1-F | CTGGCTCAGAGAATGTAACAA | 60 |
|  | c64912.graph\_c1-R | GATTGTGTTGCCAACTTCTTT |  |
| EIN3-binding F-box protein | c85195.graph\_c0-F | GAGGTGGATAGTAGACATCAAC | 60 |
|  | c85195.graph\_c0-R | CAATCGTTTATGTTCTCAGGTG |  |
| CAP | c69462.graph\_c0-F | GTGAATCTTGAGGTAGTCTTGT | 60 |
|  | c69462.graph\_c0-R | CCAATGATCTCACATCTCCTT |  |
| indole-3-acetic acid-amido synthetase | c82761.graph\_c0-F | GTGGTTGGTACTTTGGTAGCA | 60 |
|  | c82761.graph\_c0-R | TCTTCATCTCCCTAACATAATGGC |  |
| SAUR-like auxin-responsive family protein | c63578.graph\_c0-F | TCTTTGAAATTGAGGATGAGGCA | 60 |
|  | c63578.graph\_c0-R | GAGTCTTCCTGAAGATGTACCAAA |  |
| flavanone 3-hydroxylase | c70484.graph\_c0-F | TGCTAGGATGAAGAAGCTAG | 60 |
|  | c70484.graph\_c0-R | AATCTCATTCAAAGGCTTGG |  |
| shikimate hydroxycinnamoyl transferase | c83150.graph\_c0-F | TGGTGTATGGAAATTCGGTAC | 60 |
|  | c83150.graph\_c0-R | AGAGATTCGACAATGGTGC |  |
| chalcone reductase | c85005.graph\_c0-F | ATTCCAACCACAGGCATCT | 60 |
|  | c85005.graph\_c0-R | TGAACCTTCCCATCTCAACA |  |
| chalcone and stilbene synthase family protein | c67970.graph\_c0-F | GACTCACAATCGAGACTGTTG | 60 |
|  | c67970.graph\_c0-R | GGGAAAGTAGAATTTGTTGCCT | 60 |
|  |  |  |  |

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Table S4 Positive ion patterns of metabolites expressed by VIP > 1 and P < 0.05 in alfalfa based on analysis of variance** | | | | | | | | | | | | | | | |
|  | name | ZS11 | ZS22 | ZS33 | HX11 | HX22 | HX33 | HX\_vs\_ZS\_log2FC | HX\_vs\_  ZS\_Pvalue | HX\_vs\_  ZS\_VIP | HX\_vs\_ZS\_  (FC\_1\_Pvalue\_0.05\_VIP\_1)  \_regulated | HMDB\_  axonomy | sub\_class  (HMDB) | pubchem\_compound\_id  (HMDB) | cas\_registry\_number  (HMDB) |
| pos\_13 | Lumichrome | 0.000109568 | 0.000202367 | 0.000160064 | 0.000261349 | 0.000265327 | 0.00034681 | -0.888000818 | 0.025847217 | 1.617397741 | down | -- | -- | -- | -- |
| pos\_14 | 6-Hydroxypyridine-3-carboxylic acid | 4.25E-06 | 9.73E-06 | 8.82E-06 | 1.79E-05 | 2.14E-05 | 1.34E-05 | -1.210488203 | 0.029967759 | 1.615325308 | down | Pyridines and derivatives | Pyridinecarboxylic acids and derivatives | 72924 | 5006-66-6 |
| pos\_19 | Kynurenine | 4.44E-05 | 4.63E-05 | 3.73E-05 | 5.85E-05 | 5.88E-05 | 5.28E-05 | -0.409866748 | 0.017531939 | 1.683587226 | down | Organooxygen compounds | Carbonyl compounds | 161166 | 2922-83-0 |
| pos\_41 | Guanosine | 0.000284529 | 0.000361289 | 0.000403259 | 0.000468249 | 0.000649155 | 0.000514819 | -0.637718681 | 0.048304488 | 1.553497954 | down | -- | -- | -- | -- |
| pos\_46 | MG(0:0/18:3(6Z,9Z,12Z)/0:0) | 0.001684591 | 0.002326765 | 0.003143895 | 0.003972548 | 0.005341891 | 0.004180884 | -0.915385223 | 0.024379927 | 1.615610066 | down | -- | -- | -- | -- |
| pos\_56 | JUGLONE | 1.01E-05 | 2.50E-05 | 1.71E-05 | 3.52E-05 | 3.64E-05 | 4.75E-05 | -1.189361483 | 0.018798354 | 1.657060653 | down | -- | -- | -- | -- |
| pos\_91 | 3,5,7-Trimethyl-2E,4E,6E,8E-decatetraene | 2.90E-06 | 3.74E-06 | 5.26E-06 | 6.63E-06 | 8.46E-06 | 6.72E-06 | -0.875086633 | 0.023013136 | 1.6247772 | down | -- | -- | -- | -- |
| pos\_106 | Pinolenic Acid | 0.000419638 | 0.000579948 | 0.000748869 | 0.000938179 | 0.001273558 | 0.000984236 | -0.8701744 | 0.027506311 | 1.603263482 | down | -- | -- | -- | -- |
| pos\_117 | Styrene | 6.74E-06 | 8.07E-06 | 8.22E-06 | 1.08E-05 | 9.01E-06 | 1.02E-05 | -0.378721178 | 0.030309863 | 1.585806269 | down | -- | -- | -- | -- |
| pos\_145 | Cycloseychellene | 2.12E-06 | 2.02E-06 | 1.71E-06 | 2.79E-06 | 4.12E-06 | 3.95E-06 | -0.891176868 | 0.048384481 | 1.652024863 | down | -- | -- | -- | -- |
| pos\_177 | (S)-gamma-Calacorene | 3.97E-06 | 4.82E-06 | 5.59E-06 | 6.91E-06 | 1.02E-05 | 8.69E-06 | -0.844066706 | 0.03922407 | 1.625963247 | down | -- | -- | -- | -- |
| pos\_200 | 3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one | 5.28E-06 | 5.98E-06 | 5.78E-06 | 6.50E-06 | 7.30E-06 | 7.07E-06 | -0.292366877 | 0.016134426 | 1.674951201 | down | -- | -- | -- | -- |
| pos\_238 | (2xi,20beta)-2,20-Dihydroxy-3-oxo-12-ursen-28-oic acid | 0.000388551 | 0.000339379 | 0.00041765 | 0.000713313 | 0.000640886 | 0.000585789 | -0.759970338 | 0.006473153 | 1.755310874 | down | -- | -- | -- | -- |
| pos\_243 | alpha,beta-Didehydrotryptophan | 3.73E-05 | 3.46E-05 | 2.51E-05 | 6.04E-05 | 6.46E-05 | 4.99E-05 | -0.849324652 | 0.011335811 | 1.703594661 | down | -- | -- | -- | -- |
| pos\_248 | Norecasantalol | 1.21E-06 | 2.34E-06 | 2.40E-06 | 4.13E-06 | 3.65E-06 | 3.71E-06 | -0.950041925 | 0.028353694 | 1.69658937 | down | -- | -- | -- | -- |
| pos\_254 | 1-Naphthylmethanol | 6.88E-06 | 7.94E-06 | 8.33E-06 | 1.03E-05 | 8.76E-06 | 1.00E-05 | -0.331740594 | 0.037525887 | 1.552914574 | down | Naphthalenes | \_ | 20908 | \_ |
| pos\_271 | Aplotaxene | 2.42E-06 | 4.49E-06 | 6.74E-06 | 8.96E-06 | 1.15E-05 | 9.27E-06 | -1.121693812 | 0.029688324 | 1.625917142 | down | -- | -- | -- | -- |
| pos\_277 | LysoPE(18:3(6Z,9Z,12Z)/0:0) | 8.54E-05 | 0.00011366 | 0.000114898 | 0.000206436 | 0.000249439 | 0.000235924 | -1.139652395 | 0.001847346 | 1.803860917 | down | Glycerophospholipids | Glycerophosphoethanolamines | 52925136 | \_ |
| pos\_299 | Pubescenol | 0.003189227 | 0.003975056 | 0.004823171 | 0.006165329 | 0.005633186 | 0.005231949 | -0.50659246 | 0.049537452 | 1.553232171 | down | -- | -- | -- | -- |
| pos\_345 | Indole-3-acetamide | 3.92E-06 | 4.71E-06 | 4.38E-06 | 6.00E-06 | 6.31E-06 | 6.20E-06 | -0.508088211 | 0.008033575 | 1.800007237 | down | Indoles and derivatives | Indoles | 397 | 879-37-8 |
| pos\_364 | 3-Indoleacetonitrile | 1.46E-05 | 1.66E-05 | 1.74E-05 | 2.70E-05 | 2.99E-05 | 2.36E-05 | -0.72625134 | 0.015570809 | 1.739122376 | down | Indoles and derivatives | Indoles | 351795 | 771-51-7 |
| pos\_367 | LysoPC(18:3(6Z,9Z,12Z)) | 0.000171787 | 0.000486388 | 0.000326502 | 0.000719936 | 0.00099125 | 0.000802342 | -1.351992184 | 0.014090743 | 1.690069252 | down | Glycerophospholipids | Glycerophosphocholines | 52924045 | \_ |
| pos\_398 | 26-(2-Glucosyl-6-acetylglucosyl]-1,3,11,22-tetrahydroxyergosta-5,24-dien-26-oate | 0.002716262 | 0.002701927 | 0.003183093 | 0.004252464 | 0.003326996 | 0.004108365 | -0.442382854 | 0.04940355 | 1.552005406 | down | -- | -- | -- | -- |
| pos\_414 | 4,4'-Diapo-zeta-carotene | 1.96E-05 | 3.81E-05 | 3.69E-05 | 4.94E-05 | 6.58E-05 | 5.49E-05 | -0.846152158 | 0.032673253 | 1.595544808 | down | -- | -- | -- | -- |
| pos\_448 | Soyasaponin II | 0.000309045 | 0.000290015 | 0.00040857 | 0.00071074 | 0.000776047 | 0.000673261 | -1.100097288 | 0.00149258 | 1.797708244 | down | Prenol lipids | Terpene glycosides | 13326381 | 55319-36-3 |
| pos\_494 | alpha-licanic acid | 2.91E-05 | 3.70E-05 | 3.91E-05 | 4.85E-05 | 5.02E-05 | 4.43E-05 | -0.443025742 | 0.033812025 | 1.624270726 | down | -- | -- | -- | -- |
| pos\_518 | Otobanone | 1.15E-05 | 1.43E-05 | 1.84E-05 | 2.01E-05 | 2.66E-05 | 2.15E-05 | -0.625908968 | 0.046490471 | 1.520007569 | down | Aryltetralin lignans | \_ | 14462032 | 34426-79-4 |
| pos\_621 | Methyl jasmonate | 0.000470966 | 0.000542307 | 0.000390418 | 0.000687916 | 0.000576681 | 0.000693346 | -0.480113892 | 0.034411088 | 1.582182364 | down | -- | -- | -- | -- |
| pos\_755 | Pro Trp Trp Ile | 0.00041796 | 0.000512226 | 0.000606073 | 0.000777773 | 0.000733704 | 0.000774575 | -0.573436913 | 0.036925088 | 1.689432305 | down | -- | -- | -- | -- |
| pos\_782 | (3R,2'S)-Myxol 2'-伪-L-fucoside | 0.000377487 | 0.000421281 | 0.000441807 | 0.000560672 | 0.000620792 | 0.000557005 | -0.486807426 | 0.004188121 | 1.761528272 | down | -- | -- | -- | -- |
| pos\_872 | Phe Phe His Tyr | 6.03E-05 | 6.67E-05 | 7.52E-05 | 0.000119755 | 0.000113114 | 0.00011417 | -0.779833321 | 0.00253387 | 1.817853736 | down | -- | -- | -- | -- |
| pos\_920 | Hypusine | 4.75E-06 | 4.28E-06 | 5.22E-06 | 7.00E-06 | 7.32E-06 | 7.44E-06 | -0.610341118 | 0.004030466 | 1.79745035 | down | -- | -- | -- | -- |
| pos\_939 | 1-Nitrosonaphthalene | 8.11E-06 | 1.22E-05 | 1.28E-05 | 1.83E-05 | 2.87E-05 | 2.55E-05 | -1.129144869 | 0.033885432 | 1.652980085 | down | -- | -- | -- | -- |
| pos\_1003 | Gly Arg Asp Tyr | 4.90E-05 | 6.04E-05 | 7.01E-05 | 9.22E-05 | 8.22E-05 | 9.87E-05 | -0.606345305 | 0.017630935 | 1.658200979 | down | -- | -- | -- | -- |
| pos\_1066 | Pro Tyr | 3.61E-05 | 3.33E-05 | 4.40E-05 | 5.96E-05 | 6.21E-05 | 4.99E-05 | -0.597398856 | 0.017624336 | 1.646605921 | down | -- | -- | -- | -- |
| pos\_1078 | N-Acetyl-leu-leu-tyr-amide | 0.000111374 | 0.000151522 | 0.000303684 | 0.000465159 | 0.000755262 | 0.000598767 | -1.682941271 | 0.018849556 | 1.66705385 | down | -- | -- | -- | -- |
| pos\_1141 | Bilirubin | 5.12E-05 | 5.93E-05 | 6.52E-05 | 0.000100693 | 8.58E-05 | 1.00E-04 | -0.705528714 | 0.004708658 | 1.752131717 | down | Tetrapyrroles and derivatives | Bilirubins | 5315454 | 69853-44-7 |
| pos\_1159 | 5-methoxyuridine | 1.29E-05 | 1.40E-05 | 1.53E-05 | 1.76E-05 | 2.02E-05 | 2.16E-05 | -0.49396055 | 0.019648466 | 1.678393446 | down | -- | -- | -- | -- |
| pos\_1188 | 9Z,12Z-Linoleic acid | 1.26E-05 | 1.62E-05 | 2.00E-05 | 2.44E-05 | 3.36E-05 | 2.53E-05 | -0.773037611 | 0.038689989 | 1.572080414 | down | Fatty Acyls | Lineolic acids and derivatives | 5280450 | 60-33-3 |
| pos\_1322 | Terfenadine | 0.000279194 | 0.000241209 | 0.000284397 | 0.00032754 | 0.000330639 | 0.000321274 | -0.28334495 | 0.045706093 | 1.658536085 | down | -- | -- | -- | -- |
| pos\_1350 | Alanyl-Methionine | 2.23E-06 | 1.59E-06 | 9.67E-07 | 3.88E-06 | 3.67E-06 | 3.50E-06 | -1.209365168 | 0.021472505 | 1.745693397 | down | Carboxylic acids and derivatives | Amino acids, peptides, and analogues | 85744 | 14486-05-6 |
| pos\_12 | Rosmarinic acid | 4.72E-05 | 4.34E-05 | 7.30E-05 | 1.85E-05 | 7.11E-06 | 2.67E-05 | 1.644111608 | 0.03649255 | 1.614572392 | up | Cinnamic acids and derivatives | Hydroxycinnamic acids and derivatives | 5281792 | 20283-92-5 |
| pos\_47 | Formononetin | 0.000314048 | 0.00027658 | 0.000280587 | 8.06E-05 | 3.81E-05 | 0.000110518 | 1.926228227 | 0.002462868 | 1.814169277 | up | -- | -- | -- | -- |
| pos\_223 | Trp Glu His Trp | 0.001211858 | 0.001317333 | 0.00111503 | 0.001027575 | 0.001055465 | 0.000924739 | 0.276911747 | 0.046843133 | 1.526130194 | up | -- | -- | -- | -- |
| pos\_314 | Formononetin 7-O-glucoside-6''-malonate | 0.002504985 | 0.002152386 | 0.002202093 | 0.000577682 | 0.000227619 | 0.000620394 | 2.266430883 | 0.000434724 | 1.829434688 | up | Isoflavonoids | Isoflavonoid O-glycosides | 131750877 | 34232-16-1 |
| pos\_415 | Iloprost | 0.000746404 | 0.00083003 | 0.000720728 | 0.000660704 | 0.000665862 | 0.000580096 | 0.268802374 | 0.040716252 | 1.529535075 | up | -- | -- | -- | -- |
| pos\_577 | cGMP | 7.11E-05 | 6.44E-05 | 4.39E-05 | 3.96E-05 | 1.71E-05 | 3.18E-05 | 1.018768952 | 0.047511881 | 1.521482753 | up | Purine nucleotides | Cyclic purine nucleotides | 24316 | 7665-99-8 |
| pos\_603 | Fabianine | 0.00100484 | 0.001077935 | 0.001067847 | 0.000634592 | 0.000423562 | 0.000685652 | 0.85339685 | 0.021333223 | 1.750380975 | up | -- | -- | -- | -- |
| pos\_672 | 4',5'-Dihydroxy-7-methoxy-5,2'-oxido-4-phenylcoumarin | 8.12E-05 | 0.00014324 | 0.00015306 | 3.78E-05 | 7.14E-06 | 6.20E-05 | 1.820880664 | 0.035803831 | 1.582475849 | up | -- | -- | -- | -- |
| pos\_675 | Apigenin 4'-[feruloyl-(->2)-glucuronyl-(1->2)-glucuronide] | 0.031080874 | 0.022542221 | 0.027420637 | 0.013204234 | 0.014132577 | 0.019624663 | 0.787222919 | 0.02509893 | 1.631637495 | up | -- | -- | -- | -- |
| pos\_693 | 5-(3',5')-Dihydroxyphenyl-gamma-valerolactone | 5.67E-05 | 4.71E-05 | 5.62E-05 | 1.71E-05 | 8.63E-06 | 3.29E-05 | 1.450016769 | 0.026576638 | 1.695855242 | up | -- | -- | -- | -- |
| pos\_718 | 5,2'-Dihydroxy-6,7,6'-trimethoxyflavanone | 1.44E-05 | 1.59E-05 | 1.16E-05 | 6.69E-06 | 4.49E-06 | 9.07E-06 | 1.047469383 | 0.01721437 | 1.645252881 | up | -- | -- | -- | -- |
| pos\_751 | Ser Leu Ile | 4.94E-05 | 4.01E-05 | 4.44E-05 | 2.83E-05 | 2.79E-05 | 2.09E-05 | 0.797707169 | 0.006569011 | 1.741965628 | up | -- | -- | -- | -- |
| pos\_760 | Ile Phe | 9.00E-05 | 6.77E-05 | 8.10E-05 | 5.82E-05 | 5.28E-05 | 3.55E-05 | 0.704961048 | 0.031008743 | 1.596163082 | up | -- | -- | -- | -- |
| pos\_787 | NH-DVal(NMe)-Val-OMe | 0.000213756 | 0.000158215 | 0.000181901 | 0.000131278 | 0.000120359 | 6.82E-05 | 0.792144902 | 0.038331254 | 1.568745175 | up | -- | -- | -- | -- |
| pos\_811 | Anhydroamarouciaxanthin B | 6.73E-05 | 8.28E-05 | 7.06E-05 | 4.57E-05 | 5.18E-05 | 5.10E-05 | 0.572538955 | 0.023494721 | 1.694628961 | up | -- | -- | -- | -- |
| pos\_817 | Ser Trp His Trp | 0.000123633 | 0.000115847 | 9.48E-05 | 6.34E-05 | 5.02E-05 | 5.96E-05 | 0.948922219 | 0.013138869 | 1.747578687 | up | -- | -- | -- | -- |
| pos\_857 | Met Arg Trp Cys | 0.000579043 | 0.000598932 | 0.000450269 | 0.000393866 | 0.000315021 | 0.00041049 | 0.540620658 | 0.046367241 | 1.546165174 | up | -- | -- | -- | -- |
| pos\_891 | 3-O-Methylniveusin A | 0.00015932 | 0.000136649 | 0.000170633 | 0.000104421 | 9.68E-05 | 7.96E-05 | 0.732692656 | 0.009367487 | 1.735513532 | up | -- | -- | -- | -- |
| pos\_895 | BENDIOCARB | 0.000317835 | 0.00025554 | 0.000261804 | 0.000197658 | 0.000167413 | 0.000161488 | 0.665488699 | 0.018072422 | 1.705773424 | up | -- | -- | -- | -- |
| pos\_897 | ent-15-Oxo-16-kauren-19-oic acid | 0.000106478 | 0.000111076 | 9.53E-05 | 8.12E-05 | 8.38E-05 | 7.30E-05 | 0.393989823 | 0.015508557 | 1.675331113 | up | -- | -- | -- | -- |
| pos\_917 | Pro Leu | 0.000262924 | 0.00020301 | 0.000228259 | 0.000173164 | 0.000167634 | 0.000109694 | 0.623834931 | 0.039571095 | 1.561178649 | up | -- | -- | -- | -- |
| pos\_945 | 17-keto-4(Z),7(Z),10(Z),13(Z),15(E),19(Z)-Docosahexaenoic Acid | 0.000258679 | 0.000270412 | 0.000245397 | 0.000231601 | 0.00022282 | 0.000201756 | 0.239158619 | 0.027727865 | 1.595189451 | up | -- | -- | -- | -- |
| pos\_967 | Prengenine | 9.81E-05 | 0.000113576 | 6.70E-05 | 4.05E-05 | 2.38E-05 | 4.56E-05 | 1.342821573 | 0.036612955 | 1.620646938 | up | -- | -- | -- | -- |
| pos\_994 | 12-hydroxy-10-dodecenoic acid | 4.87E-05 | 4.41E-05 | 5.41E-05 | 3.54E-05 | 3.56E-05 | 3.41E-05 | 0.484576001 | 0.03719965 | 1.725848889 | up | -- | -- | -- | -- |
| pos\_1008 | 12-oxo-LTB4 | 1.76E-05 | 1.72E-05 | 1.50E-05 | 1.27E-05 | 1.35E-05 | 9.86E-06 | 0.46544797 | 0.032372424 | 1.586885633 | up | Fatty Acyls | Eicosanoids | 5280876 | 136696-10-1 |
| pos\_1012 | Uridine | 1.05E-05 | 1.10E-05 | 9.25E-06 | 7.38E-06 | 6.97E-06 | 6.16E-06 | 0.585958604 | 0.008276726 | 1.73034762 | up | -- | -- | -- | -- |
| pos\_1059 | 2-hexadecenal | 1.06E-05 | 9.34E-06 | 6.37E-06 | 2.99E-06 | 3.48E-06 | 2.94E-06 | 1.485103042 | 0.043858321 | 1.686276834 | up | Fatty Acyls | Fatty aldehydes | 5280541 | \_ |
| pos\_1131 | 4-Methylumbelliferyl beta-D-glucuronide | 0.00011106 | 0.000102042 | 0.000110123 | 7.08E-05 | 6.17E-05 | 8.50E-05 | 0.571742234 | 0.022066804 | 1.720792244 | up | -- | -- | -- | -- |
| pos\_1164 | 16b-Hydroxyestradiol | 1.20E-05 | 1.23E-05 | 1.15E-05 | 1.11E-05 | 1.04E-05 | 1.06E-05 | 0.155010693 | 0.01882982 | 1.636395642 | up | Steroids and steroid derivatives | Estrane steroids | 68929 | 547-81-9 |
| pos\_1168 | Tylosin | 0.000227458 | 0.000173674 | 0.000194525 | 0.000129397 | 0.000133434 | 0.000147854 | 0.536453799 | 0.046033621 | 1.645809134 | up | Organooxygen compounds | Carbohydrates and carbohydrate conjugates | 6260974 | 1401-69-0 |
| pos\_1203 | Linoleoyl ethanolamide | 0.000206253 | 0.000204208 | 0.00019482 | 0.000103501 | 0.00014967 | 0.000121022 | 0.693822701 | 0.023224702 | 1.739518057 | up | Organonitrogen compounds | Amines | 5283449 | \_ |
| pos\_1231 | Lysyl-Valine | 1.18E-05 | 8.79E-06 | 8.45E-06 | 6.50E-06 | 6.19E-06 | 2.77E-06 | 0.909653851 | 0.048055439 | 1.520134551 | up | Carboxylic acids and derivatives | Amino acids, peptides, and analogues | 6992341 | 20556-11-0 |
| pos\_1293 | Harpagoside | 0.000209671 | 0.000193416 | 0.00019948 | 5.10E-05 | 4.34E-05 | 9.90E-05 | 1.639735684 | 0.011450738 | 1.796726841 | up | -- | -- | -- | -- |

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| **Table S5 Negative ion patterns of metabolites represented by VIP > 1 and P < 0.05 in alfalfa based on analysis of variance** | | | | | | | | | | | | | | | |
| #ID | name | ZS11 | ZS22 | ZS33 | HX11 | HX22 | HX33 | HX\_vs\_ZS\_log2FC | HX\_vs\_ZS\_Pvalue | HX\_vs\_ZS\_VIP | HX\_vs\_ZS\_(FC\_1\_Pvalue\_0.05\_VIP\_1)\_regulated | HMDB\_taxonomy | sub\_class(HMDB) | pubchem\_compound\_id(HMDB) | cas\_registry\_number(HMDB) |
| neg\_6 | N-Acetyl-L-phenylalanine | 0.002198598 | 0.002216964 | 0.00287984 | 0.003201432 | 0.003300295 | 0.003441423 | -0.446715561 | 0.048400314 | 1.688756529 | down | -- | -- | -- | -- |
| neg\_125 | N-.alpha.-Acetyl-L-arginine | 2.59E-05 | 1.96E-05 | 2.56E-05 | 3.11E-05 | 3.27E-05 | 2.95E-05 | -0.392787295 | 0.049537639 | 1.557088617 | down | -- | -- | -- | -- |
| neg\_131 | Phorbol 12,13-dibutanoate | 0 | 4.21E-06 | 4.44E-06 | 4.29E-05 | 2.51E-05 | 4.18E-05 | -3.664958525 | 0.022453822 | 1.794529715 | down | -- | -- | -- | -- |
| neg\_137 | Sissotrin | 0.000265206 | 0.000364716 | 0.000502254 | 0.000679102 | 0.000807225 | 0.000606837 | -0.886587357 | 0.024859666 | 1.631591969 | down | -- | -- | -- | -- |
| neg\_143 | n-Propyl cinnamate | 1.45E-05 | 1.40E-05 | 1.38E-05 | 1.72E-05 | 1.66E-05 | 1.71E-05 | -0.268245743 | 0.000589611 | 1.829709567 | down | Cinnamic acids and derivatives | Cinnamic acid esters | 86306394 | 7778-83-8 |
| neg\_152 | Floxuridine | 0.000346541 | 0.000357551 | 0.000206382 | 0.000849376 | 0.000944055 | 0.000690711 | -1.448056539 | 0.006340817 | 1.740207911 | down | Pyrrolopyrazines | \_ | 11018843 | 274-45-3 |
| neg\_249 | Leukotriene E4 | 0 | 0 | 0 | 0.000292614 | 0.000372297 | 0.000294468 | #NAME? | 0.006674306 | 1.838986627 | down | -- | -- | -- | -- |
| neg\_262 | Coproporphyrin I | 0.000615123 | 0.001158505 | 0.001589248 | 0.002428986 | 0.002206845 | 0.002208689 | -1.025253844 | 0.046479549 | 1.706595378 | down | Tetrapyrroles and derivatives | Porphyrins | 440776 | 15569-30-9 |
| neg\_333 | Doxycycline | 1.19E-05 | 2.43E-05 | 6.39E-05 | 8.75E-05 | 0.000105015 | 0.000109145 | -1.592568938 | 0.03525091 | 1.709140293 | down | -- | -- | -- | -- |
| neg\_348 | Histidinyl-Glutamine | 0.000139051 | 0.00022525 | 0.000290002 | 0.000751739 | 0.000551212 | 0.000712203 | -1.622861221 | 0.005263334 | 1.805908919 | down | Carboxylic acids and derivatives | Amino acids, peptides, and analogues | 9900427 | 224638-13-5 |
| neg\_362 | Histidinyl-Glycine | 6.65E-05 | 0.000101678 | 3.62E-05 | 0.000193867 | 0.00021034 | 0.000145926 | -1.428076698 | 0.013039885 | 1.665947604 | down | Carboxylic acids and derivatives | Amino acids, peptides, and analogues | 101179 | 2578-58-7 |
| neg\_376 | 1-Stearoyl-2-hydroxy-sn-glycero-3-phosphocholine | 6.69E-05 | 9.98E-05 | 0.000101063 | 0.000134347 | 0.000198577 | 0.000197158 | -0.985651995 | 0.034747879 | 1.684793719 | down | Glycerophospholipids | Glycerophosphocholines | 497299 | 19420-57-6 |
| neg\_401 | Pyruvate | 0 | 9.94E-07 | 7.58E-06 | 1.06E-05 | 1.28E-05 | 1.69E-05 | -2.234103918 | 0.026999725 | 1.681296929 | down | Keto acids and derivatives | Alpha-keto acids and derivatives | 1060 | 127-17-3 |
| neg\_404 | Genistein | 4.87E-07 | 1.02E-05 | 3.85E-05 | 7.34E-05 | 4.97E-05 | 7.52E-05 | -2.01323313 | 0.028121416 | 1.676755475 | down | -- | -- | -- | -- |
| neg\_443 | N-Acetylglucosaminylasparagine | 1.40E-05 | 2.11E-05 | 2.35E-05 | 4.83E-05 | 4.46E-05 | 5.26E-05 | -1.31134896 | 0.001654646 | 1.850273315 | down | Organooxygen compounds | Carbohydrates and carbohydrate conjugates | 123826 | 2776-93-4 |
| neg\_445 | Alanyl-Glutamine | 5.07E-05 | 5.40E-05 | 4.56E-05 | 0.000109235 | 0.00011136 | 0.000149891 | -1.301138191 | 0.027549654 | 1.796571984 | down | Carboxylic acids and derivatives | Amino acids, peptides, and analogues | 123935 | 39537-23-0 |
| neg\_490 | Astemizole | 0.000234224 | 0.000654126 | 0.000567535 | 0.001177239 | 0.001795258 | 0.001685103 | -1.677689498 | 0.013189108 | 1.752866132 | down | -- | -- | -- | -- |
| neg\_494 | 2-Deoxy-D-glucose 6-phosphate | 5.73E-05 | 5.28E-05 | 5.35E-05 | 6.57E-05 | 6.52E-05 | 6.12E-05 | -0.23083995 | 0.009089613 | 1.668996415 | down | -- | -- | -- | -- |
| neg\_513 | Isoleucyl-Valine | 0.000156648 | 0.000165918 | 0.000156556 | 0.000178766 | 0.000178023 | 0.000181653 | -0.168397953 | 0.015291641 | 1.805407188 | down | -- | -- | -- | -- |
| neg\_170 | Flutamide | 0.00023338 | 0.000425461 | 0.000358104 | 3.10E-05 | 7.21E-07 | 0.000111405 | 2.828873774 | 0.017855362 | 1.653938237 | up | -- | -- | -- | -- |
| neg\_181 | Azelaic acid | 0.000102576 | 0.000113437 | 0.000144905 | 4.97E-05 | 4.50E-05 | 3.62E-05 | 1.463029581 | 0.01892503 | 1.760005403 | up | -- | -- | -- | -- |
| neg\_233 | Daidzein | 0.00244368 | 0.003719306 | 0.001876748 | 0.000328492 | 0 | 0.000488979 | 3.297907868 | 0.040069035 | 1.676107514 | up | -- | -- | -- | -- |
| neg\_324 | Cytidine 5'-monophosphate (CMP) | 0.000407617 | 0.00043899 | 0.000279155 | 0.000160149 | 4.20E-05 | 7.87E-05 | 2.003381831 | 0.011932102 | 1.735400494 | up | -- | -- | -- | -- |
| neg\_352 | 3-Dehydroshikimic acid | 3.11E-05 | 2.93E-05 | 1.59E-05 | 5.28E-06 | 2.94E-06 | 1.43E-05 | 1.762084665 | 0.044179547 | 1.551269434 | up | -- | -- | -- | -- |
| neg\_353 | Uridine 5'-monophosphate (UMP) | 0.000116264 | 0.000114896 | 6.08E-05 | 5.39E-05 | 2.49E-05 | 1.14E-05 | 1.694342164 | 0.045133769 | 1.609579873 | up | -- | -- | -- | -- |
| neg\_388 | D-Mannose-6-phosphate | 9.23E-05 | 0.000117535 | 6.32E-05 | 3.61E-05 | 1.14E-05 | 1.79E-05 | 2.060144642 | 0.03103848 | 1.678195963 | up | -- | -- | -- | -- |
| neg\_487 | Tryptophyl-Glutamate | 0.003872443 | 0.002662996 | 0.002871149 | 0.001356355 | 0.001444398 | 0.001486444 | 1.133636966 | 0.043384439 | 1.75144528 | up | -- | -- | -- | -- |
| neg\_509 | Sulfamethoxazole | 0.00026532 | 0.00024335 | 0.000231144 | 1.40E-05 | 1.31E-06 | 1.77E-05 | 4.488122739 | 0.000272042 | 1.869627565 | up | -- | -- | -- | -- |