**Supporting information**

**RNA and protein biomarkers for detecting enhanced metabolic resistance to herbicides mesosulfuron-methyl and fenoxaprop-ethyl in black-grass (*Alopecurus myosuroides*)**

**Running title: Herbicide resistance biomarkers in black-grass**

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**Supplementary method**

Quantitative real-time PCR

RT-qPCR was performed in Light Cycler 96 system (Roche, United Kingdom) in total volume of 20 µL containning 5 µL of cDNA, 2 µL of 0.5 µM forward primer, 2 µL of 0.5 µM reverse primer, and 10 µL LightCycler FastStart DNA Master SYBR Green I (Roche, United Kingdom). The specific primer sequences are listed in Suppplmentary Table S1. The reactions were run in the three-step programme including metling curve, pre-incubation at 95°C, 3 minutes, amplification for 40 cycles (95°C for 10 seconds, 60°C for 10 seconds and 72°C for 20 seconds minutes), and melting curve analysis from 65°C to 95°C. The reference gene used for normallisation is the blackgrass glycerealdehype-3-phosphate dehydrogenase (AmGAPPH, accession number: JN599100). The relative transcript expression (2-ΔΔCt) was calculated based on Livak and Schimitten, 2001 and Pfalffl, 2001.

**Supplementary Table S1** Specific primer sequences for qRT-PCR of 24 candidate genes identified in NTSR in black-grass

|  |  |  |  |
| --- | --- | --- | --- |
| Contig | Primer ID | Forward (5’ 3’) | Reverse (5’ 3’) |
| R00041432 | CYP450s | AAGCACCCCAATGCCTTGT | TCGCGAAGTTCTGCCCTATG |
| R00030509 | CYP450s | AACCGCGGACGTGATCTC | AGACCTTTTTCCCTTCCGTGTAG |
| R00027925 | CYP450s | TCCCCTAAGTACGTCCATGC | CTACATCTCCGGGCTCTTCA |
| R000277289 | Isoflavone hydroxylase | ATGGCTGCATCCACCATGT | AGAGTGACGATGATGGGCAAGTTC |
| R002332027 | OPR1 | GGTACCTCATCGAGCAGTTCCT | TTTTCAAGGCTACCACCGTACTC |
| R00052495 | OPR1 | GGGCCGCAGATTAGTTTTGA | GTCATCGACTATCCCGGGAAT |
| Rm00002116 | OPR1 | ATCCGTCTCTCCCCCTTCAC | TCGTTGAGCACGGTAGACATG |
| R00029421 | Carboxyl esterase | GGCGACGTCGAGTTCTACGA | AGCTCCTTGGCGGCATTC |
| R00029215 | Zeatin UGT | GCAGCAAGCAGAGGTTCATCT | TCGCCGGACTCTGCAAAT |
| R00007921 | GSTU6-like | GCCAACAAGAGCGAGCTTCT | TGGATGAGCACCGGTATCTTC |
| R00030700 | GSTU6-like | ACTCCCTCGGGTATCTCGATCT | GACCGTCATGCCGAACATC |
| R00005793 | GSTU6-like | TTTGTCAGCAGGGTGAAACTTG | TTGTGCACCGGGTTGGA |
| R00096975 | GSTU6-like | TCCCTGGTCATCGTGCAGTA | GGGTCGGAGGAAAGCAATG |
| R00029476 | GSTF1 | AGCATAAGAGCCCCGAGCAC | CCGTCCTGGAAAGCAGGGATTTG |
| R00010869 | Aminotransferase | GCATTFCAACCGTTTGTTGT | TGTATCTTCTTTGCCTGGTTGACT |
| R00029959 | Cellulose synthase | ACGTGGGATTGCGACATGTTC | CAGCAGCAGGCACATAAGCAT |
| Rm00043661 | ABC transporter | TGTGGTGCAGGAAATGGTATTTT | TGGTCTGCTGCCCTGCAT |
| R00030815 | MATE transporter | TCCACAACCTCTCTGTGCTG | TGGGAACTCCGACCAAGTAG |
| R0000345 | Thiol methyl transferase | ACCTCATGTACCTGCCTCAA | TCGAGCACCGTGGTCTTGT |
| Rm00016513 | Thiol methyl transferase | ACCCTCATGTACCTGCCTCAA | TCGAGCACCGTGGTGTTGT |
| Rm00004119 | Thiol methyl transferase | CCCTCATGTACCTGCCTCAAG | CATAGTCGAGCACTGTGGTGTTG |
| R00029303 | Pathogenesis related protein | ACAGTCTCATCAACGAAGTCTTAGCTA | GTGGCGTGTCGAAGTGGAA |
| R00003857 | Pathogenesis related protein | GCTTCGCCATCGAGGTGAT | GTACCCCAGTGACGGAACTT |
| R00004163 | Gag-pol retrotransposon | AGATCGTCGAGTATCAACCGTATG | TTTGACGTTCCGCCTTAAGAG |
| Reference | Glyceraldehype 3 phosphate dehydrogenase  | ACTGATCGAACATCTTFATGC | GACCATCCACAGTCTTCTGG |

**Supplementary Table S2** HPLC gradient timings for mesosulfuron

|  |  |  |
| --- | --- | --- |
| Time (m) | % Solvent A | % Solvent B |
| 1.0 | 80 | 20 |
| 8.0 | 0 | 100 |
| 10.0 | 80 | 20 |

**Supplementary Table S3** HPLC gradient timings for fenoxaprop

|  |  |  |
| --- | --- | --- |
| Time (m) | % Solvent A | % Solvent B |
| 1.0 | 80 | 20 |
| 1.5 | 25 | 75 |
| 8.0 | 0 | 100 |
| 10.0 | 80 | 20 |

**Supplementary Figure S1** Fenoxaprop chromatograms from a susceptible plant (top) or a resistant plant (bottom), fenoxaprop acid retention time is between 5min 8secs and 5min 44secs

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**Supplementary Figure S2** Mesosulfuron chromatograms from a susceptible plant (top) or a resistant plant (bottom), mesosulfuron-methyl retention time is between 4min 54secs and 5min 22secs

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**Supplementary Table S4** Specific primer sequences for qRT-PCR of six biomarkers for detection of NTSR in black-grass

|  |  |  |
| --- | --- | --- |
| Primer ID | Forward (5´ 3´) | Reverse (5´ 3´) |
| *AmGSTF1* | CCGAGTACGAGGTGGTGAAC | CGTCCTGGAAAGCAGGGATT |
| *AmGSTU2* | TTGACCCCGTTAAGACTCCC | GCAAACTCGACCAACCTGTC |
| *AmCYP450* | TTCTCCTTCTCCCTGTCCCC | TCAGGACCATGGGAAGACCA |
| *AmOPR1* | TTGAGATCCATGGCGCGAAT | CAAGGCTACCACCGTACTCG |
| *AmUGT* | GCAGCAAGCAGAGGTTCATCT | TCGCCGGACTCTGCAAAT |
| *AmABC* | TGTGGTGCAGGAAATGGTATTTT | TGGTCTGCTGCCCTGCAT |
| *AmGADPH* | CCCTCAAGCAAGGACTGGAG | AGCTTGCCATTGAACTCAGGA |
| *AmUBQ* | AGAAGACCTACACCAAGCCC | AGTAGTGGCGGTCGAAGTG |

**Supplementary Table S5** qRT-PCR cycling conditions

|  |  |  |  |
| --- | --- | --- | --- |
| Step | Temperature °C | Time | No. of cycles |
| Takyon activation | 95 | 3 min. | 1 |
| Denaturation | 95 | 3 sec. | 40 |
| Annealing | 60 | 15 sec. | 40 |
| Extension | 72 | 15 sec. | 40 |

**Supplementary Table S6** Pyrosequencing conditions and primer sequences for analysis of herbicide target-site mutations

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**Supplementary Table S7** Linear regression models with basal transcript expression regressed against fenoxaprop acid. The normalised basal expression of each biomarker was used as independent variable to predict the remaining radiolabelled fenoxaprop at 16 h after treatment in black-grass population.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Model | Response variable | Explanatory variable | Estimate | S.E. | N | R2 | Adjusted R2 | F(1,28) | *P*-value |
| 1 | Fenoxaprop acid | *AmGSTF1* | -0.04 | 0.01 | 30 | 0.37 | 0.35 | 16.67 | <0.001\*\* |
| 2 | Fenoxaprop acid | *AmGSTU2* | -0.02 | 0.01 | 30 | 0.18 | 0.15 | 6.24 | <0.001\*\* |
| 3 | Fenoxaprop acid | *AmCYP450* | -0.00 | 0.02 | 30 | 0.00 | -0.03 | 0.04 | 0.83 |
| 4 | Fenoxaprop acid | *AmOPR1* | -0.03 | 0.01 | 30 | 0.29 | 0.26 | 11.24 | 0.002\*\* |
| 5 | Fenoxaprop acid | *AmUGT* | -0.03 | 0.02 | 30 | 0.02 | -0.01 | 0.64 | 0.43 |
| 6 | Fenoxaprop acid | *AmABC* | 0.01 | 0.03 | 30 | 0.01 | -0.03 | 0.15 | 0.70 |

\*\* significant difference

**Supplementary Table S8** Linear regression models with basal transcript expression regressed against mesosulfuron. The normalised basal expression of each biomarker was used as independent variable to predict the remaining radiolabelled mesosulfuron at 16 h after treatment in black-grass population.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Model | Response variable | Explanatory variable | Estimate | S.E. | N | R2 | Adjusted R2 | F(1,28) | *P*-value |
| 1 | Mesosulfuron | *AmGSTF1* | -0.06 | 0.04 | 30 | 0.07 | 0.03 | 1.96 | 0.17 |
| 2 | Mesosulfuron | *AmGSTU2* | -0.08 | 0.04 | 30 | 0.17 | 0.14 | 5.72 | <0.001\*\* |
| 3 | Mesosulfuron | *AmCYP450* | -0.04 | 0.07 | 30 | 0.01 | -0.02 | 0.42 | 0.52 |
| 4 | Mesosulfuron | *AmOPR1* | -0.10 | 0.03 | 30 | 0.23 | 0.25 | 10.62 | 0.003\*\* |
| 5 | Mesosulfuron | *AmUGT* | -0.12 | 0.07 | 30 | 0.10 | 0.07 | 3.08 | 0.09 |
| 6 | Mesosulfuron | *AmABC* | 0.02 | 0.11 | 30 | 0.00 | -0.03 | 0.03 | 0.86 |

\*\* significant difference

**Supplementary Table S9** Linear regression models with protein expression regressed against fenoxaprop acid. The basal abundance level of each protein biomarker was used as independent variable to predict the remaining radiolabelled fenoxaprop at 16 h after treatment in black-grass population. The ability to significantly predict the metabolism of fenoxaprop of each biomarker gene was based the significant of F-test for linear regression.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Model | Response variable | Explanatory variable | Estimate | S.E. | N | R2 | Adjusted R2 | F(1,28) | *P*-value |
| 1 | Fenoxaprop acid | *AmGSTF1* | -0.00045 | 0.00014 | 30 | 0.27 | 0.25 | 10.53 | 0.003\*\* |
| 2 | Fenoxaprop acid | *AmGSTU2* | -0.00017 | 0.000041 | 30 | 0.38 | 0.36 | 17.15 | <0.001\*\* |
| 3 | Fenoxaprop acid | *AmOPR1* | -0.17 | 0.070 | 30 | 0.17 | 0.14 | 5.67 | 0.02\*\* |

\*\* significant difference

**Supplementary Table S10** Linear regression models with protein expression regressed against mesosulfuron. The basal abundance level of each protein biomarker was used as independent variable to predict the remaining radiolabelled mesosulfuron at 16 h after treatment in black-grass population.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Model | Response variable | Explanatory variable | Estimate | S.E. | N | R2 | Adjusted R2 | F(1,28) | *P*-value |
| 1 | Mesosulfuron | *AmGSTF1* | -0.0016 | 0.00052 | 30 | 0.26 | 0.23 | 11.96 | 0.002\*\* |
| 2 | Mesosulfuron | *AmGSTU2* | -0.00056 | 0.00016 | 30 | 0.31 | 0.28 | 14.31 | <0.001\*\* |
| 3 | Mesosulfuron | *AmOPR1* | 0.040 | 0.28 | 30 | 0.020 | 0.89 | 0.02 | 0.89 |

\*\* significant difference