

<110> FarmHannong Co., Ltd.

<120> Variantes de la protoporfirinógeno oxidasa y métodos y composiciones para conferir y/o mejorar la tolerancia a herbicidas utilizando las mismas

<130> OPP20173125KR

<150> KR 10-2016-0075357

<151> 2016-06-16

<160> 164

<170> Kopatent In 2.0

<210> 1

<211> 486

<212> PRT

<213> *Oscillatoria nigroviridis* PCC 7112

<220>

<221> PÉPTIDO

<222> (1)..(486)

<223> CyPP02

<400> 1

Met Glu Leu Leu Asp Thr Leu Ile Val Gly Ala Gly Ile Ser Gly Leu
1 5 10 15

Ser Leu Ala His Ala Leu His Lys Glu Ala Thr Ser Ala Ser Pro Leu
20 25 30

Lys Ile Leu Val Ala Glu Ser Gln Gly Arg Val Gly Gly Asn Ile Thr
35 40 45

Thr Val Thr Ala Glu Gly Phe Leu Trp Glu Glu Gly Pro Asn Ser Phe
50 55 60

Ser Pro Thr Pro Glu Leu Met Lys Leu Ala Val Asp Val Gly Leu Lys
65 70 75 80

Gln Glu Leu Ile Phe Ala Asp Arg Lys Leu Pro Arg Phe Val Tyr Trp
85 90 95

Glu Asn Lys Leu Gln Pro Val Pro Met Thr Pro Pro Ala Met Ile Gln
100 105 110

Ser Gln Leu Leu Ser Phe Pro Gly Lys Leu Arg Ala Leu Phe Gly Ala
115 120 125

Leu Gly Phe Val Ala Pro Ala Met Gly Asp Arg Leu Ser Gln Gln Gly
130 135 140

Asn Glu Glu Thr Val Ser Gln Phe Phe Arg Arg His Leu Gly Thr Glu

145		150		155		160
Val Met Gln Arg Leu	Val Glu Pro Phe	Val Ser Gly Val Tyr	Ala Gly			
	165	170	175			
Asp Pro Gln Gln Leu Ser	Ala Ala Ala Phe Gly Arg	Val Ala Lys				
	180	185	190			
Met Ala Asp Val Gly Gly Gly	Leu Val Ala Gly Ala Leu	Leu Ser Ala				
	195	200	205			
Lys Asn Arg Pro Lys Lys	Met Pro Ala Asp Pro Asn	Val Pro Lys Thr				
	210	215	220			
Lys Pro Gly Glu Leu Gly	Ser Phe Lys Gln Gly Leu Lys	Ala Leu Pro				
	225	230	235			240
Glu Ala Ile Ala Ala Lys	Leu Gly Asp Arg Val Lys	Leu Asn Trp His				
	245	250				
Leu Thr Arg Leu Gln Arg Thr	Glu Arg Glu Thr Tyr Ile	Ala Glu Phe				
	260	265	270			
Ser Thr Pro Asp Gly Gln Gln	Glu Val Glu Ala Arg Thr	Val Val Leu				
	275	280	285			
Thr Thr Pro Ala Tyr Val Thr	Ala Asp Leu Leu Gln Pro	Leu Glu Pro				
	290	295	300			
Gln Val Ser Ser Ala Leu Gln	Ala Phe Thr Tyr Pro Thr	Val Ala Ser				
	305	310	315			320
Val Val Leu Ala Tyr Pro Gln	Ser Asp Val Lys Gly Lys Leu	Val Gly				
	325	330	335			
Phe Gly Asn Leu Ile Pro Arg	Gly Gln Gly Ile Arg Cys	Leu Gly Thr				
	340	345	350			
Ile Trp Thr Ser Ser Leu Phe	Pro Asp Arg Ala Pro Ala	Gly Trp Gln				
	355	360	365			
Thr Leu Thr Ser Tyr Ile Gly	Gly Ala Thr Asp Ser Glu	Ile Gly Asn				
	370	375	380			
Leu Asp Ser Glu Gln Ile Val	Arg Glu Val His Arg Asp	Leu Ser Arg				
	385	390	395			400
Ile Leu Leu Lys Pro Asp Val	Pro Gln Pro Lys Val Leu	Thr Val Lys				
	405	410	415			
Leu Trp Lys Arg Ala Ile Pro	Gln Tyr Asn Leu Gly His	Phe Asp Arg				
	420	425	430			
Leu Gln Gln Ile Asp Glu Gly	Leu Lys Ser Leu Pro Gly	Val Tyr Leu				

435		440		445
Cys Ser Asn Tyr Val Gly Gly Val Ala Leu Gly Asp Cys Val Arg Arg				
450		455		460
Gly Phe Asp Arg Ala Arg Glu Val Gly Glu Tyr Leu Gln Lys Lys Gln				
465		470		475
				480
Ser Asp Thr Arg Ser Ile				
	485			

<210> 2
 <211> 1461
 <212> ADN
 <213> *Oscillatoria nigroviridis* PCC 7112

<220>
 <221> gene
 <222> (1)..(1461)
 <223> CyPP02

<400> 2

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gcacttcaca aggaagcaac gaggatcatcg ccgctgaaga ttttagtcgc tgagagtcag	120
ggacgtgtgg gcgggaacat cagcactgtg acagcagagg ggtttctctg ggaggagggc	180
ccgaacagtt tttcgccgac gccggaattg atgaagtggg ctgtggatgt gggattgaag	240
caggagttga tttttgccga tcgcaaattg cctcgttttg tgtattggga aaataagctg	300
caaccggtgc cgatgactcc accggcgatg attcagtctc agttgctgag ttttcggggg	360
aaactgcggg cgttgttcgg ggctttgggg tttgtcgcgc cggcaatggg cgatcgactt	420
tcgcagcagg gtaacagga aacagtttct caatttttc gccgtcatct cggtagcgaa	480
gtgatgcagc ggttgggtga accttttgtt tctggggttt atgccggcga tccgcaacaa	540
cttagcgcgg cggcggcttt tggccgggta gccaaagtgg ctgatgtggg tggcgggctg	600
gtggcggggg cgctgctttc tgctaaaaac agaccgaaga aaatgcctgc agaccggaat	660
gttcctaaaa ctaagccggg ggagttgggt tcgttcaagc aggggttgaa ggctttgcc	720
gaggcgatcg ctgctaagtt gggcgatcga gtgaaactca actggcactt gactcgctc	780
cagcgcacag aacgcgaaac ttacattgct gaattctcga cgcccgacgg acagcaggaa	840
gttgaggcgc gcaccgtggt ttgacaacg cccgcttacg ttacagccga tttgttgcaa	900

cctctggaac cgcaagttag cagcgcttta caagctttta cttatcctac ggttgcctcc 960
gttgtcttag cataccgcga gtcggatgtc aagggtaaat tagtgggttt tggaaattta 1020
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gatcgcgcg ctcgagggtg gcaaactctc accagttaca tcggcggggc aacagactcg 1140
gaaattggca atctcgactc agaacaaatc gttcgggagg tacaccgaga tttgtctcgg 1200
atthtctga aaccagatgt gccacagcca aaagttttaa cggatgaagct gtggaaacgg 1260
gcatctctc agtacaattt ggggcatttc gatcgctgc aacaaatcga tgagggtta 1320
aaatctttgc ctggagtga tttgtgcagc aactacgttg gcggagtggc tttgggagat 1380
tgcgtgcgaa ggggtttcga tcgtgcgcga gaagtggcg agtatttgca gaagaaacaa 1440
tcagatactc gatcgatctg a 1461

<210> 3
<211> 479
<212> PRT
<213> Lyngbya sp. PCC 8106

<220>
<221> PÉPTIDO
<222> (1)..(479)
<223> CyPP04

<400> 3
Met Thr His Val Leu Asp Ser Leu Ile Val Gly Ala Gly Ile Ser Gly
1 5 10 15
Leu Ala Leu Ala His Ala Leu His Gln Asn Gln Asp His Gln Leu Pro
20 25 30
Leu Asn Ile Leu Val Ser Glu His Gln Gly Arg Val Gly Gly Asn Ile
35 40 45
Thr Thr Val Ser Glu Gly Glu Phe Leu Trp Glu Glu Gly Pro Asn Ser
50 55 60
Phe Ser Pro Thr Pro Glu Leu Leu Lys Leu Ala Val Glu Val Gly Leu
65 70 75 80
Lys Pro Glu Leu Val Phe Ala Asp Arg Lys Leu Pro Arg Tyr Val Tyr
85 90 95
Trp Asn Gly Gln Leu Met Pro Val Pro Met Ser Pro Pro Ala Leu Leu
100 105 110

Ser Thr Lys Leu Leu Ser Pro Gly Gly Lys Leu Arg Ala Leu Thr Gly
 115 120 125
 Ala Leu Gly Phe Val Gln Pro Ala Met Gly Glu Ser Leu Ser Gln Gln
 130 135 140
 Asn Gly Glu Glu Thr Ile Ser Gln Phe Phe Glu Arg His Leu Gly Ser
 145 150 155 160
 Glu Val Leu Lys Arg Leu Val Glu Pro Phe Val Ser Gly Val Tyr Ala
 165 170 175
 Gly Asp Pro Gln Gln Leu Glu Ile Ser Ser Ala Phe Ala Arg Val Ala
 180 185 190
 Arg Met Ala Tyr Ser Gly Gly Gly Leu Val Ala Gly Ala Val Leu Ser
 195 200 205
 Arg Arg Gln Asn Lys Ser Pro Arg Ser Pro Ala Asp Pro Ser Ile Pro
 210 215 220
 Gln Thr Lys Arg Gly Glu Leu Gly Ser Phe Arg Gln Gly Ile Gly Ala
 225 230 235 240
 Leu Pro Asn Ala Ile Ala Lys Gln Leu Gly Asp Gln Leu Lys Leu Asn
 245 250 255
 Trp Gln Leu Thr Arg Leu Glu Arg Thr Glu Asn Gln Thr Tyr Arg Ala
 260 265 270
 Glu Phe Ser Thr Pro Glu Gly Val Gln Gln Val Glu Thr Arg Thr Val
 275 280 285
 Val Leu Thr Thr Pro Ala Tyr Val Thr Ala Glu Ile Leu Lys Pro Leu
 290 295 300
 Gln Leu Gln Val Ser Gln Thr Leu Thr Glu Ile Pro Tyr Pro Pro Val
 305 310 315 320
 Ala Cys Val Val Leu Ala Tyr Pro Val Ser Ala Leu Lys Gln Lys Leu
 325 330 335
 Thr Gly Phe Gly Asn Leu Val Pro Arg Gly Gln Gly Ile Arg Thr Leu
 340 345 350
 Gly Thr Ile Trp Thr Ser Ser Leu Phe Pro Gly Arg Ala Pro Gln Gly
 355 360 365
 Trp Gln Val Leu Thr Ser Tyr Ile Gly Gly Ala Thr Asp Pro Glu Ile
 370 375 380
 Gly Glu Leu Glu Asp Asp Gln Ile Val Glu Ala Val His Gln Asp Leu
 385 390 395 400

Arg His Ile Leu Leu Lys Glu Asp Ile Ser Pro Lys Val Leu Ala Val
405 410 415

His Leu Trp Lys Arg Ala Ile Pro Gln Tyr Asn Leu Gly His Gln Gln
420 425 430

Arg Leu Gln His Val Asn Glu Gly Leu Glu Ala Met Pro Gly Leu Tyr
435 440 445

Leu Cys Ser Asn Tyr Ile Asp Gly Val Ala Leu Gly Asp Cys Val Arg
450 455 460

Arg Ser Ile Gly Gln Ala Asn Glu Ile Leu Ser Phe Leu Gly Gln
465 470 475

<210> 4
<211> 1440
<212> ADN
<213> Lyngbya sp. PCC 8106

<220>
<221> gene
<222> (1)..(1440)
<223> CyPP04

<400> 4
atgactcacg tactcgatag tttaatcgtc ggtgcaggca ttagcggcct ggcgttagct 60
catgctctcc atcagaacca agatcatcaa ttgcctctca acattcttgt cagcgagcat 120
caaggacggg taggaggaaa tataaccaca gtatccgaag gagaatttct ttgggaagaa 180
ggccccaata gtttttctcc aaccctgag ttactgaagt tagcggtaga agtaggtctt 240
aagcctgagc tagtctttgc cgatcgcaag ttacctcggc acgtttactg gaatggtcaa 300
ctcatgcctg tgccgatgag tcctccggct ttgttagta caaaactctt aagtcctgga 360
ggtaaacttc gagcattaac gggggcattg gggtttgtac aaccgcgat gggagaatcg 420
ttaagtcaac aaaatgggga agaaacgac tcgcagtttt ttgagcgtca tttgggttca 480
gaagttctca agcgactggt tgaacccttt gtttctggtg tttatgcagg cgatccccag 540
caactcgaaa ttagctcggc ttttcccga gtcgcacgta tggcttacag tggcgggtgga 600
ttggttgctg gagcggtttt atcgcgtcgt cagaacaaat ctccgcgac gcctgccgac 660
ccgtctattc cccaaactaa acggggagag ttgggtctt ttcgtcaggg gattggagcc 720
ttaccaatg cgatcgccaa acagttaggc gatcaactca aattaaactg gcaactcacc 780

cgtctcgaac ggactgaaaa ccaaacctat cgggctgaat ttctgactcc agaaggggtt 840
 caacaggtag aaactcgaac ggtggtgttg acgactccgg cctatgtcac agcagaaatt 900
 ctcaaaccgt tgcaactcca agtcagtcaa acgttaactg aaattcccta tccccgggtg 960
 gcttgcgtcg ttttagccta tcccgtttca gctttaaagc agaaattaac cggatttggc 1020
 aatttagttc cccgaggaca agggattcgg acgttaggca cgatttggac atcgagtta 1080
 tttctggtc gcgccccca aggctggcaa gtcctacca gttatatgg cggagcgacg 1140
 gatccagaaa ttggagagtt agaagatgat caaattgttg aggcggttca tcaagattg 1200
 cgtcacattt tactcaaaga agatatctct cccaaagtgc tagccgtgca tctgtgga 1260
 cgtgctatcc ctcaatacaa tctcggacac caacaacggt tacaacacgt taatgagggt 1320
 ctagaggcaa tgccgggggt atatctgtgt agcaactata tcgacggtgt agcgttggga 1380
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 1440

<210> 5
 <211> 466
 <212> PRT
 <213> Halotheca sp. PCC 7418

<220>
 <221> PÉPTIDO
 <222> (1)..(466)
 <223> CyPP08

<400> 5
 Met Ile Asp Thr Leu Ile Val Gly Ala Gly Ile Ser Gly Leu Ser Ala
 1 5 10 15
 Ala Tyr Arg Leu Asp Glu Lys Gln Arg Gln Val Leu Val Ala Glu Lys
 20 25 30
 Arg Asp Arg Ala Gly Gly Asn Ile Thr Ser Gln Gln Ser Gly Asp Phe
 35 40 45
 Leu Trp Glu Glu Gly Pro Asn Ser Phe Ser Pro Thr Pro Glu Leu Leu
 50 55 60
 Lys Leu Ala Val Asp Ala Gly Leu Arg Asn Glu Leu Ile Phe Ala Asp
 65 70 75 80
 Arg Gly Leu Pro Arg Tyr Val Tyr Trp Glu Gly Lys Leu Arg Pro Val
 85 90 95

Pro Met Ser Pro Pro Thr Ala Val Thr Ser Gln Leu Leu Ser Pro Ile
 100 105 110

Gly Lys Leu Arg Ala Leu Thr Gly Ala Leu Gly Phe Ile Pro Pro Gln
 115 120 125

Val Ser Ser Gln Glu Glu Thr Val Ala Asp Phe Phe Thr Arg His Leu
 130 135 140

Gly Ser Glu Val Ala Gln Arg Leu Val Ser Pro Phe Val Ser Gly Val
 145 150 155 160

Tyr Ala Gly Asp Val Asp Gln Leu Ser Ala Glu Ala Ala Phe Gly Arg
 165 170 175

Val Thr Gln Leu Ala Asp Val Gly Gly Gly Leu Val Ala Gly Ala Ile
 180 185 190

Leu Cys Arg Arg Gln Lys Pro Lys Ser Thr Pro Lys Thr Ala Lys Pro
 195 200 205

Ser Asp Ile Pro Glu Thr Lys Ser Gly Gln Leu Gly Ser Phe Lys Glu
 210 215 220

Gly Leu Gln Gln Leu Pro Ser Ala Ile Val Ser Gln Leu Gly Asp Lys
 225 230 235 240

Val Lys Phe Gln Trp Glu Leu Lys Asn Ile Ser Pro His Pro Glu Ser
 245 250 255

Gly Tyr Val Ala Thr Phe Ser Thr Pro Glu Gly Glu Gln Thr Val Glu
 260 265 270

Ala Lys Thr Val Ile Leu Thr Thr Pro Ala Tyr Val Thr Ala Ser Leu
 275 280 285

Val Lys Asp Leu Ser Pro Gln Ala Ser Gln Ala Leu Asn Glu Ile Ser
 290 295 300

Tyr Pro Pro Val Ala Cys Val Val Leu Ala Tyr Pro Asp Glu Ala Leu
 305 310 315 320

Arg Phe Pro Leu Lys Gly Phe Gly Asn Leu Asn Pro Arg Ser Gln Gly
 325 330 335

Ile Arg Thr Leu Gly Thr Ile Trp Ser Ser Thr Leu Phe Pro Gly Arg
 340 345 350

Thr Pro Lys Gly Trp His Leu Leu Thr Asn Phe Ile Gly Gly Ala Thr
 355 360 365

Asp Pro Ala Ile Ala Glu Leu Ser Glu Asp Gln Ile Ile Glu Gln Val
 370 375 380

His Gln Asp Leu Gln Gln Ala Val Ile Lys Ser Gly Ser Ile Pro Lys
385 390 395 400

Pro Leu Ala Val His Leu Trp Ser Lys Ala Ile Pro Gln Tyr Asn Leu
405 410 415

Gly His Leu Lys Arg Leu Glu Thr Ile Arg Asn His Leu Lys Pro Phe
420 425 430

Ser Gly Leu Phe Leu Ser Ser Asn Tyr Leu Asp Gly Val Ala Leu Gly
435 440 445

Asp Cys Val Arg Arg Gly Glu Glu Ser Ser Gln Ala Val Leu Asp Tyr
450 455 460

Leu Gly
465

<210> 6
<211> 1401
<212> ADN
<213> Halotheca sp. PCC 7418

<220>
<221> gene
<222> (1)..(1401)
<223> CyPP08

<400> 6
atgatagata ctttaattgt gggagcaggg attagtgggt taagtgctgc gtatcgactc 60
gatgagaagc agcgccaagt gctggttgca gaaaagcgcg atcgcgctgg gggaaatatc 120
accagccaac aaagtggcga tttcctctgg gaagaaggac cgaacagttt ttctcccaca 180
ccagaactcc taaaactagc ggttgatgcg ggcttaagaa atgagttaat ctttgctgat 240
cgcggacttc cccgttatgt ttattgggag gggaaactgc gccctgttcc tatgagtccc 300
cccacagccg tgacatccca gttgctgagt ccaatcgga aactacgggc gttaacgggt 360
gcattaggct ttattccccc gcaagtgtcg agtcaggaag aaacgggtgc ggactttttt 420
accgtcatc tcggttcaga agtagcccaa cggttagtga gtccgtttgt gtctgggggt 480
tatgcagggg atgtggatca actcagtgcg gaagctgcat ttggacgagt tacccaactg 540
gcggtatggc gcggtggact ggtcgcaggt gcatTTTTAT gtcgtcgtca aaagccaaag 600
tcaaccccaa aaacggctaa accgtctgat attccagaaa caaagtctgg acagttaggt 660

tcatttaagg aaggattaca acaattaccc agcgcgatcg tttctcaact gggagacaaa	720
gtaaagtttc aatgggaact gaaaaatata tccccatc cagaatcggg ttacgtcgcg	780
acattttcca caccagaggg agaacaaca gtcgaagcca aaaccgttat cctcaccact	840
cccgcctacg ttaccgcctc tctggtcaaa gatttatcac ctcaagccag tcaagcctta	900
aacgaaattt cctatcccc cgtagcttgt gtggtcttag cctatcccga tgaagccctc	960
cgttttcccc tcaaaggatt tggtaatctt aaccctcgca gtcaaggaat ccgcactctt	1020
ggtacaattt ggagttcaac actctttcca ggacgcacgc cgaaagggtg gcatctctta	1080
accaatttta ttggcggtgc aactgatccc gcaattgctg aactcagtga agatcaaatt	1140
attgaacaag tccatcaaga cttacaaca gcggtgatta aatcggaag tatccgaaa	1200
cccttagccg ttcatTTgtg gtcaaaagcg attccgcaat acaatctcgg acatctgaaa	1260
cggttagaaa ccatccgcaa tcaactaaaa cccttctctg gactcttttt atcgagtaac	1320
tatctcgatg gcgttgcggtt gggtagattgt gtgcgccgag gggaagagag tagtcaagcc	1380
gtgtagatt atttgggtta a	1401

<210> 7
 <211> 537
 <212> PRT
 <213> Arabidopsis thaliana

<220>
 <221> PÉPTIDO
 <222> (1)..(537)
 <223> AtPP01 sivestre

<400> 7
 Met Glu Leu Ser Leu Leu Arg Pro Thr Thr Gln Ser Leu Leu Pro Ser
 1 5 10 15
 Phe Ser Lys Pro Asn Leu Arg Leu Asn Val Tyr Lys Pro Leu Arg Leu
 20 25 30
 Arg Cys Ser Val Ala Gly Gly Pro Thr Val Gly Ser Ser Lys Ile Glu
 35 40 45
 Gly Gly Gly Gly Thr Thr Ile Thr Thr Asp Cys Val Ile Val Gly Gly
 50 55 60
 Gly Ile Ser Gly Leu Cys Ile Ala Gln Ala Leu Ala Thr Lys His Pro
 65 70 75 80

Asp Ala Ala Pro Asn Leu Ile Val Thr Glu Ala Lys Asp Arg Val Gly
 85 90 95
 Gly Asn Ile Ile Thr Arg Glu Glu Asn Gly Phe Leu Trp Glu Glu Gly
 100 105 110
 Pro Asn Ser Phe Gln Pro Ser Asp Pro Met Leu Thr Met Val Val Asp
 115 120 125
 Ser Gly Leu Lys Asp Asp Leu Val Leu Gly Asp Pro Thr Ala Pro Arg
 130 135 140
 Phe Val Leu Trp Asn Gly Lys Leu Arg Pro Val Pro Ser Lys Leu Thr
 145 150 155 160
 Asp Leu Pro Phe Phe Asp Leu Met Ser Ile Gly Gly Lys Ile Arg Ala
 165 170 175
 Gly Phe Gly Ala Leu Gly Ile Arg Pro Ser Pro Pro Gly Arg Glu Glu
 180 185 190
 Ser Val Glu Glu Phe Val Arg Arg Asn Leu Gly Asp Glu Val Phe Glu
 195 200 205
 Arg Leu Ile Glu Pro Phe Cys Ser Gly Val Tyr Ala Gly Asp Pro Ser
 210 215 220
 Lys Leu Ser Met Lys Ala Ala Phe Gly Lys Val Trp Lys Leu Glu Gln
 225 230 235 240
 Asn Gly Gly Ser Ile Ile Gly Gly Thr Phe Lys Ala Ile Gln Glu Arg
 245 250 255
 Lys Asn Ala Pro Lys Ala Glu Arg Asp Pro Arg Leu Pro Lys Pro Gln
 260 265 270
 Gly Gln Thr Val Gly Ser Phe Arg Lys Gly Leu Arg Met Leu Pro Glu
 275 280 285
 Ala Ile Ser Ala Arg Leu Gly Ser Lys Val Lys Leu Ser Trp Lys Leu
 290 295 300
 Ser Gly Ile Thr Lys Leu Glu Ser Gly Gly Tyr Asn Leu Thr Tyr Glu
 305 310 315 320
 Thr Pro Asp Gly Leu Val Ser Val Gln Ser Lys Ser Val Val Met Thr
 325 330 335
 Val Pro Ser His Val Ala Ser Gly Leu Leu Arg Pro Leu Ser Glu Ser
 340 345 350
 Ala Ala Asn Ala Leu Ser Lys Leu Tyr Tyr Pro Pro Val Ala Ala Val
 355 360 365

Ser Ile Ser Tyr Pro Lys Glu Ala Ile Arg Thr Glu Cys Leu Ile Asp
 370 375 380
 Gly Glu Leu Lys Gly Phe Gly Gln Leu His Pro Arg Thr Gln Gly Val
 385 390 395 400
 Glu Thr Leu Gly Thr Ile Tyr Ser Ser Ser Leu Phe Pro Asn Arg Ala
 405 410 415
 Pro Pro Gly Arg Ile Leu Leu Leu Asn Tyr Ile Gly Gly Ser Thr Asn
 420 425 430
 Thr Gly Ile Leu Ser Lys Ser Glu Gly Glu Leu Val Glu Ala Val Asp
 435 440 445
 Arg Asp Leu Arg Lys Met Leu Ile Lys Pro Asn Ser Thr Asp Pro Leu
 450 455 460
 Lys Leu Gly Val Arg Val Trp Pro Gln Ala Ile Pro Gln Phe Leu Val
 465 470 475 480
 Gly His Phe Asp Ile Leu Asp Thr Ala Lys Ser Ser Leu Thr Ser Ser
 485 490 495
 Gly Tyr Glu Gly Leu Phe Leu Gly Gly Asn Tyr Val Ala Gly Val Ala
 500 505 510
 Leu Gly Arg Cys Val Glu Gly Ala Tyr Glu Thr Ala Ile Glu Val Asn
 515 520 525
 Asn Phe Met Ser Arg Tyr Ala Tyr Lys
 530 535

<210> 8
 <211> 1614
 <212> ADN
 <213> Arabidopsis thaliana

<220>
 <221> gene
 <222> (1)..(1614)
 <223> AtPP01 sivestre

<400> 8
 atggagtat ctcttctccg tccgacgact caatcgcttc ttccgtcggt ttcgaagccc 60
 aatctccgat taaatgttta taagcctctt agactccgtt gttcagtggc cgggtggacca 120
 accgtcggat cttcaaaaat cgaaggcggg ggaggcacca ccatcacgac ggattgtgtg 180
 attgtcggcg gaggtattag tggctcttgc atcgtcagg cgcttgctac taagcatcct 240

gatgctgctc cgaatttaat tgtgaccgag gctaaggatc gtgttggagg caacattatc	300
actcgtgaag agaatggttt tctctgggaa gaaggcccc atagttttca accgtctgat	360
cctatgctca ctatggtggt agatagtgtt ttgaaggatg atttggtgtt gggagatcct	420
actgcgcaa ggtttgtgtt gtggaatggg aaattgaggc cggttccatc gaagctaaca	480
gacttacgtt tctttgattt gatgagtatt ggtgggaaga ttagagctgg ttttggtgca	540
cttggcattc gaccgtcacc tccaggtcgt gaagaatctg tggaggagt tgtacggcgt	600
aacctcggtg atgaggtttt tgagcgcctg attgaaccgt tttgttcagg tgtttatgct	660
ggtgatcctt caaaactgag catgaaagca gcgtttggga aggtttggaa actagagcaa	720
aatggtggaa gcataatagg tggctactttt aaggcaattc aggagaggaa aaacgctccc	780
aaggcagaac gagacccgcg cctgccaaaa ccacagggcc aaacagttgg ttctttcagg	840
aagggacttc gaatgttgcc agaagcaata tctgcaagat taggtagcaa agttaagttg	900
tcttggaaac tctcaggtat cactaagctg gagagcggag gatacaactt aacatatgag	960
actccagatg gtttagtttc cgtgcagagc aaaagtgttg taatgacggg gccatctcat	1020
gttgcaagtg gtctcttgcg cctcttttct gaatctgctg caaatgcact ctcaaaacta	1080
tattaccac cagttgcagc agtatctatc tegtaccga aagaagcaat ccgaacagaa	1140
tgtttgatag atggtgaact aaagggtttt gggcaattgc atccacgcac gcaaggagtt	1200
gaaacattag gaactatcta cagtcctca ctctttccaa atcgcgccacc gcccgaaga	1260
attttgcgtg tgaactacat tggcgggtct acaaaccg gaattctgtc caagtctgaa	1320
ggtgagttag tggaaagcagt tgacagagat ttgaggaaaa tgctaattaa gcctaattcg	1380
accgatccac ttaaattagg agttagggtt tggcctcaag ccattcctca gtttctagtt	1440
ggtcactttg atatccttga cacggctaaa tcatctctaa cgtcttcggg ctacgaaggg	1500
ctattttttg gtggcaatta cgtcgttgtt gtagccttag gccggtgtgt agaaggcgca	1560
tatgaaaccg cgattgaggt caacaacttc atgtcacggt acgcttaca gtaa	1614

<210> 9
 <211> 537
 <212> PRT
 <213> Secuencia artificial

<220>

<223> AtPP01 tipo mutante

<400> 9

Met Glu Leu Ser Leu Leu Arg Pro Thr Thr Gln Ser Leu Leu Pro Ser
1 5 10 15

Phe Ser Lys Pro Asn Leu Arg Leu Asn Val Tyr Lys Pro Leu Arg Leu
20 25 30

Arg Cys Ser Val Ala Gly Gly Pro Thr Val Gly Ser Ser Lys Ile Glu
35 40 45

Gly Gly Gly Gly Thr Thr Ile Thr Thr Asp Cys Val Ile Val Gly Gly
50 55 60

Gly Ile Ser Gly Leu Cys Ile Ala Gln Ala Leu Ala Thr Lys His Pro
65 70 75 80

Asp Ala Ala Pro Asn Leu Ile Val Thr Glu Ala Lys Asp Arg Val Gly
85 90 95

Gly Asn Ile Ile Thr Arg Glu Glu Asn Gly Phe Leu Trp Glu Glu Gly
100 105 110

Pro Asn Ser Phe Gln Pro Ser Asp Pro Met Leu Thr Met Val Val Asp
115 120 125

Ser Gly Leu Lys Asp Asp Leu Val Leu Gly Asp Pro Thr Ala Pro Arg
130 135 140

Phe Val Leu Trp Asn Gly Lys Leu Arg Pro Val Pro Ser Lys Leu Thr
145 150 155 160

Asp Leu Pro Phe Phe Asp Leu Met Ser Ile Gly Gly Lys Ile Arg Ala
165 170 175

Gly Phe Gly Ala Leu Gly Ile Arg Pro Ser Pro Pro Gly Arg Glu Glu
180 185 190

Ser Val Glu Glu Phe Val Arg Arg Asn Leu Gly Asp Glu Val Phe Glu
195 200 205

Arg Leu Ile Glu Pro Phe Cys Ser Gly Val Tyr Ala Gly Asp Pro Ser
210 215 220

Lys Leu Ser Met Lys Ala Ala Phe Gly Lys Val Trp Lys Leu Glu Gln
225 230 235 240

Asn Gly Gly Ser Ile Ile Gly Gly Thr Phe Lys Ala Ile Gln Glu Arg
245 250 255

Lys Asn Ala Pro Lys Ala Glu Arg Asp Pro Arg Leu Pro Lys Pro Gln
260 265 270

Gly Gln Thr Val Gly Ser Phe Arg Lys Gly Leu Arg Met Leu Pro Glu
 275 280 285
 Ala Ile Ser Ala Arg Leu Gly Ser Lys Val Lys Leu Ser Trp Lys Leu
 290 295 300
 Leu Gly Ile Thr Lys Leu Glu Ser Gly Gly Tyr Asn Leu Thr Tyr Glu
 305 310 315 320
 Thr Pro Asp Gly Leu Val Ser Val Gln Ser Lys Ser Val Val Met Thr
 325 330 335
 Val Pro Ser His Val Ala Ser Gly Leu Leu Arg Pro Leu Ser Glu Ser
 340 345 350
 Ala Ala Asn Ala Leu Ser Lys Leu Tyr Tyr Pro Pro Val Ala Ala Val
 355 360 365
 Ser Ile Ser Tyr Pro Lys Glu Ala Ile Arg Thr Glu Cys Leu Ile Asp
 370 375 380
 Gly Glu Leu Lys Gly Phe Gly Gln Leu His Pro Arg Thr Gln Gly Val
 385 390 395 400
 Glu Thr Leu Gly Thr Ile Tyr Ser Ser Ser Leu Phe Pro Asn Arg Ala
 405 410 415
 Pro Pro Gly Arg Ile Leu Leu Leu Asn Met Ile Gly Gly Ser Thr Asn
 420 425 430
 Thr Gly Ile Leu Ser Lys Ser Glu Gly Glu Leu Val Glu Ala Val Asp
 435 440 445
 Arg Asp Leu Arg Lys Met Leu Ile Lys Pro Asn Ser Thr Asp Pro Leu
 450 455 460
 Lys Leu Gly Val Arg Val Trp Pro Gln Ala Ile Pro Gln Phe Leu Val
 465 470 475 480
 Gly His Phe Asp Ile Leu Asp Thr Ala Lys Ser Ser Leu Thr Ser Ser
 485 490 495
 Gly Tyr Glu Gly Leu Phe Leu Gly Gly Asn Tyr Val Ala Gly Val Ala
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 Asn Phe Met Ser Arg Tyr Ala Tyr Lys
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 aatctccgat taaatgttta taagcctctt agactccgtt gttcagtggc cgttgaccca 120

 accgtcggat ctcaaaaat cgaaggcgga ggaggc 156

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 tacgcatcat tg 72

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<400> 14
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<210> 31
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<220>
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<400> 32
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<400> 44
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<400> 45
ccggcataac acccagaaac aaaaggttcc ac 32

<210> 46
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	ctgcttgaac gaaccgaact cccccggctt agt	33
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<220>
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 gatgtccaac acgtcccag acagcgaatt c 31

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 aacggaggca gtcgtaggat aagtaaaagc 30

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aaacttctta gattcgtgta ttgggaaaac 30

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taaatttcca acaccacta attaccctt gac 33

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<400> 72
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cggcataaag cccagaaaca aaaggttcca c 31

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<400> 77
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<400> 78
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<210> 79
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<210> 89
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30

<210> 90
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30

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<210> 95
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<400> 95
tggatctcca caatacacac cagaaacaaa 30

<210> 96
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<210> 97
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<400> 97
 tggatctccc aaatacacac cagaaacaaa 30

<210> 98
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<220>
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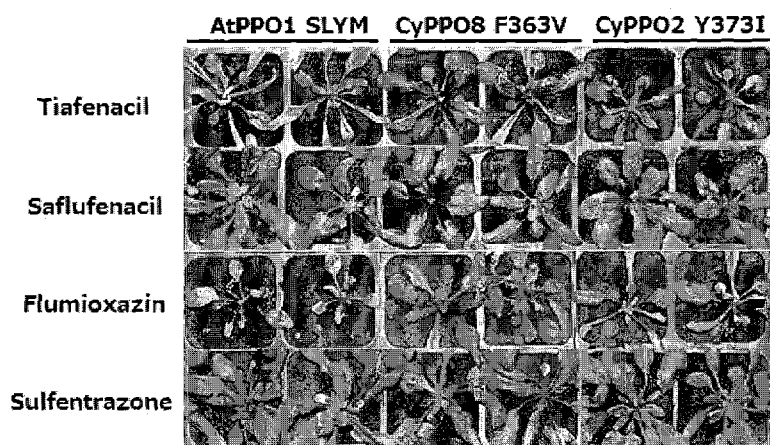
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(54) Title: PROTOPORPHYRINOGEN OXIDASE VARIANTS AND METHODS AND COMPOSITIONS FOR CONFERRING AND/OR ENHANCING HERBICIDE TOLERANCE USING THE SAME

[Fig. 45]



(57) Abstract: Provided is technology for conferring enhanced tolerance and/or enhancing tolerance to herbicide of a plant and/or algae using amino acid variants of protoporphyrinogen oxidase derived from prokaryotes.

Published:

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- with sequence listing part of description (Rule 5.2(a))

【DESCRIPTION】**【Invention Title】**

PROTOPORPHYRINOGEN OXIDASE VARIANTS AND METHODS AND
COMPOSITIONS FOR CONFERRING AND/OR ENHANCING HERBICIDE
5 TOLERANCE USING THE SAME

【Technical Field】

Provided are protoporphyrinogen oxidases derived from prokaryotes, or its
variants, and technology for conferring and/or enhancing herbicide tolerance of plants
10 and/or algae using the same.

【Background Art】

A porphyrin biosynthetic pathway serves for the synthesis of chlorophyll and
heme which play vital roles in plant metabolism, and it takes place in the chloroplast.
15 In this pathway, protoporphyrinogen IX oxidase (hereinafter, referred to as PPO;
EC:1.3.3.4) catalyzes the oxidation of protoporphyrinogen IX to protoporphyrin IX.
After the oxidation of protoporphyrinogen IX to protoporphyrin IX, protoporphyrin IX
binds with magnesium by Mg-chelatase to synthesize chlorophyll, or it binds with iron
by Fe-chelatase to synthesize heme.

20 Therefore, when PPO activity is inhibited, synthesis of chlorophylls and heme
is inhibited and the substrate protoporphyrinogen IX leaves the normal porphyrin
biosynthetic pathway, resulting in the rapid export of protoporphyrinogen IX from the
chloroplast to the cytoplasm, and cytoplasmic protoporphyrin IX accumulation caused
by the oxidation. Accumulated protoporphyrin IX generates highly reactive singlet
25 oxygen ($^1\text{O}_2$) in the presence of light and oxygen molecules which destroy cell
membrane and rapidly leads to plant cell death. Based on this principle, herbicides
inhibiting PPO activity have been developed. Until now, there have been 9 families of
PPO-inhibiting herbicides, including pyrimidinediones, diphenyl-ethers,
phenylpyrazoles, N-phenylphthalimides, thiadiazoles, oxadiazoles, triazolinones,
30 oxazolidinediones, and others herbicides, which are classified according to their

chemical structures.

Further, in order to prevent effects of these herbicides on the growth of crops while using the herbicides, there is a need to provide herbicide tolerance for the crops.

Meanwhile, algae are photosynthetic organisms that can convert light energy into chemical energy which can be used to synthesize various useful compounds. For example, algae can fix carbon by photosynthesis and convert carbon dioxide into sugar, starch, lipids, fats, or other biomolecules, thereby removing greenhouse gases from the atmosphere. In addition, large-scale cultivation of algae can produce a variety of substances such as industrial enzymes, therapeutic compounds and proteins, nutrients, commercial materials and fuel materials.

However, in case of large-scale cultivation of algae in a bioreactor or in an open or enclosed pond, contamination may occur by undesired competent organisms, for example, undesired algae, fungi, rotifer, or zooplankton.

Thus, a technology is needed to harvest desired plants and/or algae on a large scale by treating herbicides at a concentration that would inhibit the growth of competent organisms without herbicide tolerance, after conferring herbicide tolerance to desired plants and/or algae.

【References】

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(Patent document 2) U.S. patent application registration publication US 6,808,904 (2004.10.26)

(Patent document 3) U.S. patent application registration publication US 7,563,950 (2009.07.21)

(Patent document 4) International patent application laid-open publication WO2011/085221 (2011.07.14)

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【Disclosure】

【Technical Problem】

5 In this specification, it is found that hemY-type PPO genes derived from prokaryotes and mutants thereof show a broad herbicide tolerance to protoporphyrinogen oxidase (PPO)-inhibiting herbicides, and thereby it is proposed if providing plants and/or algae with the same, herbicide tolerance can be conferred and/or enhanced.

10 One embodiment provides a polypeptide variant comprising, consisting essentially of, or consisting of:

(1) an amino acid sequence wherein one or more selected from the group consisting of amino acids affecting to the interaction between PPO-inhibiting herbicides and the polypeptide of PPO, SEQ ID NO: 1 (e.g., amino acids positioned on binding sites of the polypeptide of SEQ ID NO: 1 interacting with PPO-inhibiting herbicide) are respectively independently deleted or substituted with an amino acid which is different from original amino acid in the corresponding position, or

15

(2) an amino acid sequence having 95% or higher, 98% or higher, or 99% or higher sequence homology with the amino acid sequence (1).

20 The one or more selected from the group consisting of amino acids affecting to the interaction between PPO-inhibiting herbicides and the polypeptide of PPO, SEQ ID NO: 1, may be one or more selected from the group consisting of S63, P91, R92, F169, V173, A175, E228, L229, P316, V318, F337, L340, G351, T352, I353, and Y373, of the amino acid sequence of SEQ ID NO: 1.

25 Another embodiment provides a polypeptide variant comprising, consisting essentially of, or consisting of:

(1) an amino acid sequence wherein one or more selected from the group consisting of amino acids affecting to the interaction between PPO-inhibiting herbicides and the polypeptide of PPO, SEQ ID NO: 3 (e.g., amino acids positioned on binding sites of the polypeptide of SEQ ID NO: 3 interacting with PPO-inhibiting herbicide) are

30

respectively independently deleted or substituted with an amino acid which is different from original amino acid in the corresponding position, or

(2) an amino acid sequence having 95% or higher, 98% or higher, or 99% or higher sequence homology with the amino acid sequence (1).

5 The one or more kinds selected from the group consisting of amino acids affecting to the interaction between PPO-inhibiting herbicides and the polypeptide of PPO, SEQ ID NO: 3, may be one or more selected from the group consisting of N63, S64, S66, P67, P92, R93, F170, S172, G173, V174, Y175, A176, R190, E230, L231, P318, V320, F339, G340, N341, L342, L352, G353, T354, I355, Y375, I424, V458, and
10 R465, of the amino acid sequence of SEQ ID NO: 3.

Other embodiment provides a polypeptide variant comprising, consisting essentially of, or consisting of:

(1) an amino acid sequence wherein one or more kinds selected from the group consisting of amino acids affecting to the interaction between PPO-inhibiting herbicides
15 and the polypeptide of PPO, SEQ ID NO: 5 (e.g., amino acids positioned on binding sites of the polypeptide of SEQ ID NO: 5 interacting with PPO-inhibiting herbicide), are respectively independently deleted or substituted with an amino acid which is different from original amino acid in the corresponding position, or

(2) an amino acid sequence having 95% or higher, 98% or higher, or 99% or
20 higher sequence homology with the amino acid sequence (1).

The one or more kinds selected from the group consisting of amino acids affecting to the interaction between PPO-inhibiting herbicides and the polypeptide of PPO, SEQ ID NO: 5, may be one or more selected from the group consisting of P84, R85, F156, V160, A162, Q179, P306, V308, F327, L330, I343, N362, and F363, of the
25 amino acid sequence of SEQ ID NO: 5.

Other embodiment provides a polynucleotide encoding the polypeptide variant, or a polypeptide of SEQ ID NO: 1, 3, or 5.

Other embodiment provides a recombinant vector comprising the polynucleotide.

30 Other embodiment provides a recombinant (transformed) cell comprising the

recombinant vector.

Other embodiment provides a composition for conferring or enhancing herbicide tolerance of plants or algae, comprising one or more selected from the group consisting of:

- 5 the polypeptide of SEQ ID NO: 1, the polypeptide of SEQ ID NO: 3, the polypeptide of SEQ ID NO: 5, a variant of the polypeptide, and a polypeptide with an amino acid sequence having 95% or higher, 98% or higher, or 99% or higher sequence homology with the polypeptide or the variant;

a polynucleotide encoding the polypeptide or variant;

- 10 a recombinant vector comprising the polynucleotide; and

a recombinant (transformed) cell comprising the recombinant vector.

The herbicide may be an herbicide inhibiting protoporphyrinogen oxidase.

- As a specific embodiment, the herbicide may be one or more kinds selected from the group consisting of pyrimidinediones, diphenyl-ethers, phenylpyrazoles, N-phenylphthalimides, phenylesters, thiadiazoles, oxadiazoles, triazolinones, oxazolidinediones and other herbicides, but not limited thereto.
- 15

- As a specific embodiment, the herbicide may be one or more kinds selected from the group consisting of butafenacil, saflufenacil, benzfendizone, tiafenacil, fomesafen, oxyfluorfen, aclonifen, acifluorfen, bifenox, ethoxyfen, lactofen, chlomethoxyfen, chlorintofen, fluoroglycofen-ethyl, halosafen, pyraflufen-ethyl, fluzolate, flumioxazin, cinidon-ethyl, flumiclorac-pentyl, fluthiacet, thidiazimin, oxadiargyl, oxadiazon, carfentrazone, sulfentrazone, azafenidin, pentoxazone, pyraclonil, flufenpyr-ethyl, profluazol, phenopylate (2,4-dichlorophenyl 1-pyrrolidinecarboxylate), carbamate analogues of phenopylate (for example, O-phenylpyrrolidino- and piperidinocarbamate analogues (refer to "Ujjana B. Nandihalli, Mary V. Duke, Stephen O. Duke, Relationships between molecular properties and biological activities of O-phenyl pyrrolidino- and piperidinocarbamate herbicides., J. Agric. Food Chem., 1992, 40(10) 1993-2000")), agriculturally acceptable salts thereof, and combinations thereof, but not limited thereto.
- 20
- 25

- 30 The plant means a multicellular eukaryote having photosynthetic capability,

which may be a monocotyledonous plant or a dicotyledonous plant, and may be an herbaceous plant or a woody plant. The algae mean unicellular organism having photosynthetic capability, which may be a prokaryotic algae or a eukaryotic algae.

In one embodiment, the plants and algae are genetically manipulated in order to further comprise a second herbicide tolerance polypeptide or a gene encoding thereof, and broader range of herbicide tolerance to the second herbicide may be conferred and/or enhanced. The plants and algae are genetically manipulated in order to comprise the second herbicide tolerance polypeptide or a gene encoding thereof may be prepared using a composition for conferring and/or enhancing tolerance to the herbicide wherein the second herbicide tolerance polypeptide or a gene encoding thereof is further comprised. Thus, a composition for conferring and/or enhancing tolerance to the herbicide may further comprise the second herbicide tolerance polypeptide or a gene encoding thereof.

As a specific embodiment, the second herbicide may include cell division-inhibiting herbicides, photosynthesis-inhibiting herbicides, amino acid synthesis-inhibiting herbicides, plastid-inhibiting herbicides, and cell membrane-inhibiting herbicides, but not limited thereto.

As a specific embodiment, the second herbicide may be exemplified by glyphosate, glufosinate, dicamba, 2,4-D (2,4-Dichlorophenoxyacetic acid), isoxaflutole, ALS (acetolactate synthase)-inhibiting herbicide, photosystem II-inhibiting herbicide, phenylurea-based herbicide, bromoxynil-based herbicide, and combinations thereof, but not limited thereto.

As a specific embodiment, the second herbicide may be exemplified by one or more kinds selected from the group consisting of glyphosate herbicide-tolerant EPSPS (glyphosate resistant 5-enolpyruvylshikimate-3-phosphate synthase), GOX (glyphosate oxidase), GAT (glyphosate-N-acetyltransferase) or glyphosate decarboxylase; glufosinate herbicide-tolerant PAT (phosphinothricin-N-acetyltransferase); dicamba herbicide-tolerant DMO (dicamba monooxygenase); 2,4-D herbicide-tolerant 2,4-D monooxygenase or AAD (aryloxyalkanoate dioxygenase); ALS-inhibiting sulfonylurea-based herbicide-tolerant ALS (acetolactate Synthase), AHAS (acetohydroxyacid

synthase), or athahas1 (acetohydroxyacid synthase large subunit); photosystem II-inhibiting herbicide-tolerant photosystem II protein D1; phenylurea-based herbicide-tolerant cytochrome P450; plastid-inhibiting herbicide-tolerant HPPD (hydroxylphenylpyruvate dioxygenase); bromoxynil herbicide-tolerant nitrilase; and
5 combinations thereof, but not limited thereto.

Further, the gene encoding the second herbicide-tolerant polypeptide may be exemplified by one or more kinds selected from the group consisting of glyphosate herbicide-tolerant cp4 epsps, mepsps, 2mepsps, goxv247, gat4601 or gat4621 gene; glufosinate herbicide-tolerant bar, pat or pat (SYN) gene; dicamba herbicide-tolerant
10 dmo gene; 2,4-D herbicide-tolerant AAD-1, AAD-12 gene; ALS-inhibiting sulfonylurea-based herbicide-tolerant ALS, GM-HRA, S4-HRA, ZM-HRA, Csr1, Csr1-1, Csr1-2, SurA or SurB; photosystem II-inhibiting herbicide-tolerant psbA gene; phenylurea herbicide-tolerant CYP76B1 gene; isoxaflutole herbicide-tolerant HPPDPF W336 gene and bromoxynil herbicide-tolerant bxn gene; and combinations thereof, but
15 not limited thereto.

Other embodiment provides a transformant of a plant and/or algae having herbicide tolerance, which are transformed with the polynucleotide, or a clone or progeny thereof.

Other embodiment provides a method of preparing a transgenic plant or a
20 transgenic algae having herbicide tolerance, comprising a step of transforming a plant and/or algae with the polynucleotide.

Other embodiment provides a method of conferring or enhancing herbicide tolerance of a plant and/or algae, comprising a step of transforming a plant and/or algae with the polynucleotide or a coding gene thereof.

25 The transformation may be performed on algae, and/or plant cell, protoplast, callus, hypocotyl, seed, cotyledon, shoot, or whole plant.

The transformant may be algae, and/or plant cell, protoplast, callus, hypocotyl, seed, cotyledon, shoot, or whole plant.

Other embodiment provides a method of controlling weeds in a cropland
30 comprising:

a step of providing the cropland with a plant comprising one or more selected from the group consisting of the polypeptide of SEQ ID NO: 1, 3, or 5, or the polypeptide variant, a polynucleotide encoding thereof, a recombinant vector comprising the polynucleotide, and a recombinant cell comprising the recombinant
5 vector; and

a step of applying an effective dosage of a protoporphyrinogen oxidase-inhibiting herbicide to the cropland (or to the plant).

As a specific embodiment, the step of applying an effective dosage of protoporphyrinogen oxidase-inhibiting herbicide to the cropland may be performed by
10 applying an effective dosage of two or more protoporphyrinogen oxidase-inhibiting herbicides sequentially or simultaneously.

As other embodiment, the plant may be genetically manipulated in order to further comprise a second herbicide-tolerant polypeptide or a gene encoding the same, and an effective dosage of the protoporphyrinogen oxidase-inhibiting herbicide and the
15 second herbicide may be applied sequentially or simultaneously.

Other embodiment provides a method of removing an undesired aquatic organism from a culture medium, comprising a step of providing a culture medium with algae comprising one or more selected from the group consisting of the polypeptide, the polypeptide variant, a polynucleotide encoding the polypeptide or the polypeptide
20 variant, a recombinant vector comprising the polynucleotide, and a recombinant cell comprising the recombinant vector, and a step of applying an effective dosage of a protoporphyrinogen oxidase-inhibiting herbicide to the culture medium.

【Technical Solution】

25 Provided is a technology of conferring and/or enhancing herbicide tolerance of plants or algae.

As used herein, 'conferring and/or enhancing herbicide tolerance of plants or algae' or 'enhancing herbicide tolerance of plants or algae' is interpreted as conferring tolerance on plants or algae which do not have herbicide tolerance, or enhancing
30 tolerance of plants or algae which have herbicide tolerance, or broad meaning of

covering both.

As used herein, 'consisting of a sequence,' 'consisting essentially of a sequence,' or 'comprising a sequence' is used in order to mean both cases of comprising described sequence, or necessarily comprising the sequence, and may be interpreted as
5 meaning of comprising a sequence other than described sequence and/or comprising mutation (addition, deletion, and/or substitution of an amino acid or nucleic acid), as long as maintaining an intrinsic activity of protein, polypeptide, or nucleic acid molecule and exhibiting intended function.

In one embodiment, provided are one or more polypeptide variants selected
10 from the group consisting of

a polypeptide variant comprising, consisting essentially of, or consisting of an amino acid sequence wherein one or more kinds selected from the group consisting of amino acids affecting to the interaction between PPO-inhibiting herbicides and polypeptide of PPO, SEQ ID NO: 1 (e.g., amino acids positioned on binding sites of
15 SEQ ID NO: 1 interacting with PPO-inhibiting herbicide), are respectively independently deleted or substituted with other amino acid which is different from original amino acid in the corresponding position, or an amino acid sequence having 95% or higher, 98% or higher, or 99% or higher homology thereto;

a polypeptide variant comprising, consisting essentially of, or consisting of an
20 amino acid sequence wherein one or more kinds selected from the group consisting of amino acids affecting to the interaction between PPO-inhibiting herbicides and the polypeptide of PPO, SEQ ID NO: 3 (e.g., amino acids positioned on binding sites of SEQ ID NO: 3 interacting with PPO-inhibiting herbicide), are respectively independently deleted or substituted with other amino acid which is different from
25 original amino acid in the corresponding position, or an amino acid sequence having 95% or higher, 98% or higher, or 99% or higher homology thereto;

a polypeptide variant comprising, consisting essentially of, or consisting of an amino acid sequence wherein one or more kinds selected from the group consisting of amino acids affecting to the interaction between PPO-inhibiting herbicides and the
30 polypeptide of PPO, SEQ ID NO: 5 (e.g., amino acids positioned on binding sites of

SEQ ID NO: 5 interacting with PPO-inhibiting herbicide), are respectively independently deleted or substituted with other amino acid which is different from original amino acid in the corresponding position, or an amino acid sequence having 95% or higher, 98% or higher, or 99% or higher homology thereto; and

5 combinations thereof.

In other embodiment, provided is a polynucleotide encoding the polypeptide variant, a recombinant vector comprising the polynucleotide, and a recombinant cell comprising the recombinant vector. The polynucleotide may be designed in order that an optimized codon is comprised in a cell to be transformed among codons encoding
10 each amino acid. The optimized codon may be easily known to a person skilled in the art (for example, refer to "<http://www.genscript.com/codon-opt.html>", "<http://sg.idtdna.com/CodonOpt>" etc.).

In other embodiment, provided is a composition for conferring or enhancing herbicide tolerance of plants or algae, comprising one or more kinds selected from the
15 group consisting of the polypeptide variant, a polynucleotide encoding the polypeptide, a recombinant vector comprising the polynucleotide, a recombinant cell comprising the recombinant vector, and combinations thereof.

In other embodiment, provided is a transformant having herbicide tolerance, which is transformed with the polynucleotide.

20 In other embodiment, provided is a method of preparing a transgenic plant or a transgenic algae having herbicide tolerance, comprising a step of transforming algae, or plant cell, protoplast, callus, hypocotyl, seed, cotyledon, shoot, or whole plant with the polynucleotide.

In other embodiment, provided is a method of conferring or enhancing
25 herbicide tolerance of plants or algae, comprising a step of transforming algae, or plant cell, protoplast, callus, hypocotyl, seed, cotyledon, shoot, or whole plant with the polynucleotide.

Hereinafter, the present invention will be described in more detail.

The polypeptide of an amino acid sequence of SEQ ID NO: 1, 3, 5, or its
30 variant provided herein is a PPO protein derived from a prokaryote (for example,

cyanobacteria), and is an herbicide-tolerant PPO protein having tolerance to PPO-inhibiting herbicides. Specifically, a PPO protein which is derived from *Oscillatoria nigro-viridis* PCC 7112 is provided, and it is designated as CyPPO2, and its amino acid sequence is represented by SEQ ID NO: 1, and a nucleotide sequence of a gene encoding thereof is represented by SEQ ID NO: 2. In addition, a PPO derived from *Lyngbya* sp. PCC 8106 strain is provided, and it is designated as CyPPO4, and its amino acid sequence is represented by SEQ ID NO: 3, and a nucleotide sequence of a gene encoding thereof is represented by SEQ ID NO: 4. In addition, a PPO derived from *Halotheca* sp. PCC 7418 strain is provided, and it is designated as CyPPO8, and its amino acid sequence is represented by SEQ ID NO: 5, and a nucleotide sequence of a gene encoding thereof is represented by SEQ ID NO: 6. Herein, the polypeptide and variants of polypeptide described above may be expressed respectively as herbicide-tolerant PPO protein or herbicide-tolerant PPO protein variant having tolerance to PPO-inhibiting herbicides. In addition, as used herein, "herbicide-tolerant PPO or its variant" may be used in order to mean the above herbicide-tolerant PPO protein or herbicide-tolerant PPO protein variant, herbicide-tolerant PPO protein-encoding gene or herbicide-tolerant PPO protein variant-encoding gene, or all of them.

Cyanobacteria-derived PPO proteins can have enhanced tolerance to PPO-inhibiting herbicides and/or by comprising amino acid mutation (variation) in a range of maintaining overall enzyme activity than wild type PPO proteins. Such amino acid mutation may comprise substitution, deletion, addition and/or introduction of one or more kinds of amino acids selected from amino acid residues of interaction sites between PPO proteins and herbicides.

The PPO protein variant will be described in more detail as follows.

One embodiment provides a polypeptide variant comprising, consisting essentially of, or consisting of:

an amino acid sequence wherein one or more kinds selected from the group consisting of amino acids affecting to the interaction between PPO-inhibiting herbicides and the polypeptide of PPO, SEQ ID NO: 1 (CyPPO2) (e.g., amino acids positioned in binding sites to PPO inhibiting herbicides of polypeptide of SEQ ID NO: 1), are

respectively independently deleted or substituted with other amino acid, which is different from original amino acid (namely, an amino acid in the corresponding position of the wild type), or

an amino acid sequence having 95% or higher, 98% or higher, or 99% or higher
5 homology thereto.

The amino acid residue of polypeptide of SEQ ID NO: 1, which is deleted or substituted with other amino acid that is different from original amino acid (e.g., one or more residues selected from the group consisting of amino acids positioned on binding sites to PPO-inhibiting herbicides of polypeptide of SEQ ID NO: 1), may be one or
10 more selected from the group consisting of S63(meaning of "S(Ser) at the 63rd position"; the expression of the following amino acid residues is interpreted in the same manner), P91, R92, F169, V173, A175, E228, L229, P316, V318, F337, L340, G351, T352, I353, and Y373 among the amino acid sequence of SEQ ID NO: 1.

In one specific embodiment, the variant of polypeptide may comprise, consist
15 essentially of, or consist of:

an amino acid sequence wherein one or more selected from the group consisting of S63, P91, R92, F169, V173, A175, E228, L229, P316, V318, F337, L340, G351, T352, I353, and Y373 of the amino acid sequence of SEQ ID NO: 1 are respectively and independently deleted or substituted with an amino acid which is selected from the
20 group consisting of M(Met), V(Val), I(Ile), T(Thr), L(Leu), C(Cys), A(Ala), S(Ser), F(Phe), P(Pro), W(Trp), N(Asn), Q(Gln), G(Gly), Y(Tyr), D(Asp), E(Glu), R(Arg), H(His), K(Lys), etc. and is different from the amino acid at the corresponding position in the wild type (for example, substituted with an amino acid which is selected from the group consisting of M(Met), V(Val), I(Ile), T(Thr), L(Leu), C(Cys), A(Ala), S(Ser),
25 F(Phe), etc. and is different from the amino acid at the corresponding position in the wild type), or

an amino acid sequence having 95% or higher, 98% or higher, or 99% or higher homology thereto.

For example, the variant of polypeptide may comprise, consist essentially of, or
30 consist of:

an amino acid sequence comprising one or more amino acid mutations selected from the group consisting of Y373M (meaning of “the amino acid residue at the 373rd position is substituted from Y(Tyr) to M(Met)”); the expression of the following amino acid mutations is interpreted in the same manner), Y373V, Y373I, Y373T, Y373L,
 5 Y373C, F169A, A175C, A175L, A175I, P316A, P316L, V318M, V318T, V318L, G351A, S63T, P91L, R92A, V173S, V173C, V173T, V173L, E228A, L229F, F337V, L340T, L340I, L340V, T352V, I353T, I353L, I353V, and I353C, in the amino acid sequence of SEQ ID NO: 1; or

an amino acid sequence having 95% or higher, 98% or higher, or 99% or higher
 10 homology thereto.

For example, the variant of polypeptide may comprise, consist essentially of, or consist of:

an amino acid sequence comprising one or more amino acid mutations selected from the group consisting of amino acid mutations of Y373M, Y373V, Y373I, Y373T,
 15 Y373L, Y373C, F169A, A175C, A175L, A175I, P316A, P316L, V318M, V318T, V318L, G351A, S63T, P91L, R92A, V173S, V173C, V173T, V173L, E228A, L229F, F337V, L340T, L340I, L340V, T352V, I353T, I353L, I353V, I353C, S63T+Y373M (meaning of a mutant or mutation comprising all of substitution of the 63rd residue from S to T and substitution of the 373rd residue from Y to M; the expression of the following
 20 two or more amino acid mutations is interpreted in the same manner), R92A+Y373M, V173S+Y373M, V173S+Y373I, V173S+Y373L, V173S+Y373V, V173C+Y373M, V173C+Y373I, V173T+Y373M, V173T+Y373I, V173L+Y373M, A175L+Y373M, A175L+Y373I, A175C+Y373M, A175C+Y373I, A175I+Y373M, E228A+Y373M, L229F+Y373T, V318M+Y373M, V318M+Y373I, V318M+Y373V, V318T+Y373I,
 25 L340I+Y373M, L340I+Y373I, L340V+Y373M, G351A+Y373M, I353T+Y373M, I353T+Y373I, I353T+Y373L, I353L+Y373M, I353V+Y373M, I353C+Y373M, S63T+V173S+Y373M, S36T+V173S+Y373I, S63T+I353T+Y373M, S63T+I353T+Y373I, V173S+V318M+Y373M, V173T+L340I+Y373M, V173S+A175C+Y373M, A175C+V318M+Y373M, A175L+V318M+Y373M,
 30 A175C+I353L+Y373M, A175C+I353V+Y373M, P316A+V318L, P316L+V318L,

F337V+Y373M, T352V+Y373M, G351A+T352V+Y373M, and P91L+Y373M, in the amino acid sequence of SEQ ID NO: 1, or

an amino acid sequence having 95% or higher, 98% or higher, or 99% or higher homology thereto.

5 Other embodiment provides a polypeptide variant comprising, consisting essentially of, or consisting of:

an amino acid sequence wherein one or more selected from the group consisting of amino acids affecting to the interaction between PPO-inhibiting herbicides and the polypeptide of PPO, SEQ ID NO: 3 (CyPPO4) (e.g., amino acids positioned on binding
10 sites to PPO-inhibiting herbicides of polypeptide of SEQ ID NO: 3), are respectively and independently deleted or substituted with other amino acid which is different from original amino acid (namely, an amino acid at the corresponding position of the wild type), or

an amino acid sequence having 95% or higher, 98% or higher, or 99% or higher
15 homology thereto.

The amino acid residue of polypeptide of SEQ ID NO: 3 which is deleted or substituted with other amino acid that is different from original amino acid (e.g., one or more kinds selected from the group consisting of amino acids positioned on binding sites to PPO-inhibiting herbicides of polypeptide of SEQ ID NO: 3) may be one or more
20 selected from the group consisting of N63, S64, S66, P67, P92, R93, F170, S172, G173, V174, Y175, A176, R190, E230, L231, P318, V320, F339, G340, N341, L342, L352, G353, T354, I355, Y375, I424, V458, and R465, for example, one or more selected from the group consisting of A176, P318, V320, and Y375, of the amino acid sequence of SEQ ID NO: 3.

25 In one specific embodiment, the variant of polypeptide may comprise, consist essentially of, or consist of:

an amino acid sequence wherein one or more selected from the group consisting of N63, S64, S66, P67, P92, R93, F170, S172, G173, V174, Y175, A176, R190, E230, L231, P318, V320, F339, G340, N341, L342, L352, G353, T354, I355, Y375, I424,
30 V458, and R465, for example, one or more selected from the group consisting of A176,

P318, V320, and Y375, of the amino acid sequence of SEQ ID NO: 3 are respectively and independently deleted or substituted with an amino acid which is selected from the group consisting of M(Met), V(Val), I(Ile), T(Thr), L(Leu), C(Cys), A(Ala), S(Ser), F(Phe), P(Pro), W(Trp), N(Asn), Q(Gln), G(Gly), Y(Tyr), D(Asp), E(Glu), R(Arg), H(His), K(Lys), etc. and is different from the amino acid at the corresponding position in the wild type (for example, substituted with an amino acid which is selected from the group consisting of M(Met), V(Val), I(Ile), T(Thr), L(Leu), C(Cys), A(Ala), etc. and is different from the amino acid at the corresponding position in the wild type), or

an amino acid sequence having 95% or higher, 98% or higher, or 99% or higher homology thereto.

For example, the variant of polypeptide may comprise, consist essentially of, or consist of:

an amino acid sequence comprising one or more amino acid mutations selected from the group consisting of Y375M, Y375V, Y375I, Y375T, Y375C, A176C, A176L, P318L, V320L, V320M, and P318A, in the amino acid sequence of SEQ ID NO: 3, or an amino acid sequence having 95% or higher, 98% or higher, or 99% or higher homology thereto.

More specifically, the variant of polypeptide may comprise, consist essentially of, or consist of an amino acid sequence comprising amino acid mutation of Y375M, Y375V, Y375I, Y375T, Y375C, A176C, A176L, P318L+V320L, V320M, or P318A+V320L, in the amino acid sequence of SEQ ID NO: 3 or an amino acid sequence having 95% or higher, 98% or higher, or 99% or higher homology thereto.

Other embodiment provides a polypeptide variant comprising, consisting essentially of, or consisting of:

an amino acid sequence wherein one or more selected from the group consisting of amino acids affecting to the interaction between PPO-inhibiting herbicides and the polypeptide of PPO, SEQ ID NO: 5 (CyPPO8) (e.g., amino acids positioned on binding sites to PPO-inhibiting herbicides of polypeptide of SEQ ID NO: 5), are respectively and independently deleted or substituted with other amino acid which is different from original amino acid, or

an amino acid sequence having 95% or higher, 98% or higher, or 99% or higher homology thereto.

The amino acid residue of polypeptide of SEQ ID NO: 5 which is deleted or substituted with other amino acid that is different from original amino acid (e.g., one or more kinds selected from the group consisting of amino acids positioned in binding sites to PPO-inhibiting herbicides of polypeptide of SEQ ID NO: 5) may be one or more selected from the group consisting of P84, R85, F156, V160, A162, Q179, P306, V308, F327, L330, I343, N362, and F363 of the amino acid sequence of SEQ ID NO: 5.

In one specific embodiment, the variant of polypeptide may comprise, consist essentially of, or consist of:

an amino acid sequence wherein one or more selected from the group consisting of P84, R85, F156, V160, A162, Q179, P306, V308, F327, L330, I343, N362, and F363 of the amino acid sequence of SEQ ID NO: 5 are respectively and independently deleted or substituted with an amino acid which is selected from the group consisting of M(Met), V(Val), I(Ile), T(Thr), L(Leu), C(Cys), A(Ala), S(Ser), F(Phe), P(Pro), W(Trp), N(Asn), Q(Gln), G(Gly), Y(Tyr), D(Asp), E(Glu), R(Arg), H(His), K(Lys), etc. and is different from the amino acid at the corresponding position in the wild type (for example, substituted with an amino acid which is selected from the group consisting of M(Met), V(Val), I(Ile), T(Thr), L(Leu), C(Cys), A(Ala), S(Ser), H(His), G(Gly), etc. and is different from the amino acid at the corresponding position in the wild type), or

an amino acid sequence having 95% or higher, 98% or higher, or 99% or higher homology thereto.

For example, the variant of polypeptide may comprise, consist essentially of, or consist of:

an amino acid sequence comprising one or more amino acid mutations selected from the group consisting of P84L, R85A, R85C, R85H, R85L, R85T, R85V, F363M, F363V, F363L, F363C, F363I, F363T, A162C, A162L, P306L, P306A, V308L, V308M, N362S, V160S, V160C, I343V, I343T, F156A, Q179G, F327V, and L330T, in the amino acid sequence of SEQ ID NO: 5, or

an amino acid sequence having 95% or higher, 98% or higher, or 99% or higher

homology thereto.

More specifically, the variant of polypeptide may comprise, consist essentially of, or consist of:

- an amino acid sequence comprising one or more amino acid mutations selected
- 5 from the group consisting of amino acid mutations of P84L, R85A, R85C, R85H, R85L, R85T, R85V, F363M, F363V, F363L, F363C, F363I, F363T, A162C, A162L, P306L, P306A, V308L, V308M, N362S, V160S, V160C, I343V, I343T, F156A, Q179G, F327V, L330T, P84L+F363M, R85A+F363M, R85A+F363I, R85C+F363M, R85H+F363M, R85L+F363M, R85T+F363M, R85V+F363M, R85A+A162L+F363M, R85A+A162L+F363I, R85A+A162C+F363M, R85A+A162C+F363I, R85A+V308M+F363M, V160S+F363M, V160S+F363I, V160S+V308M+F363I, A162L+F363M, A162C+F363M, A162C+F363I, A162C+F363L, A162C+V308M+F363M, A162C+V308L+F363M, A162L+Q179G+F363M, P306A+V308L, P306L+V308L, V308M+F363M, V308M+F363I, I343T+F363M, I343V+F363M, and N362S+F363M, in the amino acid sequence of SEQ ID NO: 5, or
- 10 an amino acid sequence having 95% or higher, 98% or higher, or 99% or higher homology thereto.

- The polypeptide variant comprising, consisting essentially of, or consisting of an amino acid sequence having sequence homology (for example, 95% or higher, 98% or higher, or 99% or higher sequence homology) described herein may maintain enzyme
- 20 activity equivalent to that of a polypeptide having an amino acid sequence which is a standard of identification of sequence homology (for example, the PPO protein having amino acid mutation described above), for example, 5% or higher, 10% or higher, 20% or higher, 30% or higher, 40% or higher, 50% or higher, 60% or higher, 70% or higher, 80% or higher, 90% or higher, or 95% or higher enzyme activity to a polypeptide
- 25 having an amino acid sequence which is a standard in plants (in a whole plant, in a plant cell or cell culture, in a plant tissue, etc.), in algae, and/or in vitro, and having function to confer herbicide tolerance. The sequence homology description is used in order to clarify that the herbicide-tolerance PPO protein variant or polypeptide variant described
- 30 herein may comprise all sequence mutations in the range of satisfying the above

condition (maintain enzyme activity and having function to confer herbicide tolerance).

The names of amino acids used in the description are arranged as follows:

Amino acid	3-letter code	1-letter code
Alanine	Ala	A
Isoleucine	Ile	I
Leucine	Leu	L
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Tryptophan	Trp	W
Valine	Val	V
Asparagine	Asn	N
Cysteine	Cys	C
Glutamine	Gln	Q
Glycine	Gly	G
Serine	Ser	S
Threonine	Thr	T
Tyrosine	Tyr	Y
Aspartic acid	Asp	D
Glutamic acid	Glu	E
Arginine	Arg	R
Histidine	His	H
Lysine	Lys	K

The polypeptide variant (herbicide-tolerant PPO protein variant) may maintain
 5 enzyme activity of PPO protein, and exhibit enhanced herbicide tolerance compared to the wild type.

In addition, the herbicide-tolerant PPO protein variant may comprise further mutation exhibiting biologically equal activity to a polypeptide consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, or an amino acid sequence having amino acid
 10 mutation described above. For example, the additional mutation may be amino acid substitution which does not overall alter molecular activity, and such amino acid substitution is publicly known in the art. In one example, the additional substitution may be substitution of amino acid residues Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Thr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile,
 15 Leu/Val, Ala/Glu, or Asp/Gly, but not limited thereto. In some cases, the herbicide-tolerant PPO protein variant may be under modification by one or more kinds selected from the group consisting of phosphorylation, sulfation, acylation, glycosylation, methylation, farnesylation, etc. In addition, the herbicide-tolerant PPO protein variant

may comprise a protein variant wherein structural stability to heat, pH, etc. of the protein is increased or protein activity is increased by amino acid variation (mutation) and/or modification.

The term "sequence homology" refers to the degree of similarity to the wild type or reference amino acid sequence or nucleotide sequence, and any protein may be included in the scope of the present invention, as long as it includes amino acid residues having 60% or higher, 65% or higher, 70% or higher, 75% or higher, 80% or higher, 85% or higher, 90% or higher, 95% or higher, 98% or higher, or 99% or higher identity to the amino acid sequence of the herbicide-tolerant PPO protein and retains a biological activity equivalent to the herbicide-tolerant PPO protein variant. Such protein homologues may comprise an active site equivalent to that of a targeted protein. Such homology comparison may be conducted by eye or with the aid of readily available comparison programs. Commercially available computer programs may calculate percent (%) homology between two or more sequences, and homology (%) may be calculated over contiguous sequences. The sequence alignment for comparison may be conducted by methods known in the art, for example, GAP, BESTFIT, BLAST, FASTA and TFASTA.

The herbicide-tolerant PPO protein or its variant may be obtained by extracting from nature and purifying by methods well known in the art. Otherwise, it may be obtained as a recombinant protein using a gene recombination technology. In case of using a gene recombination technology, it may be obtained by a process of collecting herbicide-tolerant PPO protein or its variant from a host cell, after introducing a nucleic acid encoding the herbicide-tolerant PPO protein or its variant into an appropriate expression vector, and transforming a host cell with the vector in order to express a targeted protein. After the protein is expressed in a selected host cell, general biochemical separation techniques, for example, treatment with a protein precipitating agent (salting out), centrifugation, ultrasonic disruption, ultrafiltration, dialysis, chromatography such as molecular sieve chromatography (gel filtration), adsorption chromatography, ion exchange chromatography, affinity chromatography and the like may be used for the isolation and purification thereof, and in order to separate the

protein with a high purity, these methods may be used in combination.

The herbicide-tolerant PPO nucleic acid molecule (polynucleotide encoding the PPO protein or its variant) may be isolated or prepared using standard molecular biological techniques, for example, a chemical synthesis or recombination method, or
5 commercially available one may be used.

In a specific embodiment, the PPO proteins were found to exhibit broad herbicide tolerance against representative 9 families of PPO inhibiting herbicides classified according to their chemical structures in a herbicide tolerance test system using PPO-deficient *E. coli* BT3(Δ PPO). It was also found that the proteins may be
10 expressed in tobacco leaves by *Agrobacterium*-mediated transformation, and they may be also expressed in the chloroplast of a plant by using a transit peptide (TP). Further, it was found that the PPO proteins may be also expressed in *Arabidopsis thaliana* ecotype Columbia-0 (*A. thaliana*) by a plant expression vector. Even though the transformed plants are treated with PPO inhibiting herbicides, germination and growth
15 of the plants are observed. Furthermore, inheritance of the above herbicide-tolerant traits to the next generation was confirmed by an inheritance study.

Therefore, the PPO protein and its variants provided herein may be introduced into a plant or an alga, thereby being used for enhancement of the herbicide tolerance of the plant or the alga.

20 One embodiment provides a composition for conferring and/or enhancing herbicide tolerance of plants and/or algae, comprising one or more selected from the group consisting of:

- (1) a polypeptide comprising, consisting essentially of, or consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, or an amino acid sequence having 95% or higher,
25 98% or higher, or 99% or higher homology thereto;
- (2) the above-described polypeptide variant;
- (3) a polynucleotide encoding the polypeptide (1) or the polypeptide variant (2);
- (4) a recombinant vector comprising the polynucleotide; and
- (5) a recombinant cell comprising the recombinant vector.

The herbicide herein refers to an active ingredient that kills, controls, or otherwise adversely modifies the growth of plants or algae. In addition, the herbicide tolerance or herbicide tolerance means that even after treatment of a herbicide which normally kills a normal or wild-type plant or normally inhibits growth thereof, inhibition of the plant growth is weakened or eliminated, compared to that of the normal or wild-type plant, and therefore, the plant continues to grow. The herbicide includes a herbicide inhibiting protoporphyrinogen oxidase (PPO) of a plant or an alga. Such PPO-inhibiting herbicide may be classified into pyrimidinediones, diphenyl-ethers, phenylpyrazoles, N-phenylphthalimides, thiadiazoles, oxadiazoles, triazolinones, oxazolidinediones, and other herbicides according to their chemical structures.

As a specific embodiment, the pyrimidinediones herbicide includes butafenacil, saflufenacil, benzfendizone, and tiafenacil, but is not limited thereto.

The diphenyl-ethers herbicide includes fomesafen, oxyfluorfen, acifluorfen, acifluorfen, bifenox, ethoxyfen, lactofen, chlomethoxyfen, chlorintofen, fluoroglycofen-ethyl and halosafen, but not limited thereto.

The phenylpyrazoles herbicide includes pyraflufen-ethyl and fluazolate, but not limited thereto.

The phenylphthalimides herbicide includes flumioxazin, cinidon-ethyl and flumiclorac-pentyl, but not limited thereto.

The phenylesters herbicide includes phenopylate (2,4-dichlorophenyl 1-pyrrolidinecarboxylate) and carbamate analogues of phenopylate (for example, O-phenylpyrrolidino- and piperidinocarbamate analogues (refer to "Ujjana B. Nandihalli, Mary V. Duke, Stephen O. Duke, Relationships between molecular properties and biological activities of O-phenyl pyrrolidino- and piperidinocarbamate herbicides., J. Agric. Food Chem., 1992, 40(10) 1993-2000"), etc., but not limited thereto. In one embodiment, the carbamate analogue of phenopylate may be one or more kinds selected from the group consisting of pyrrolidine-1-carboxylic acid phenyl ester (CAS No. 55379-71-0), 1-pyrrolidinecarboxylic acid, 2-chlorophenyl ester (CAS No. 143121-06-6), 4-chlorophenyl pyrrolidine-1-carboxylate (CAS No. 1759-02-0), carbamic acid, diethyl-, 2,4-dichloro-5-(2-propynyloxy)phenyl ester (9CI) (CAS No. 143121-07-7), 1-

pyrrolidinecarboxylic acid, 2,4-dichloro-5-hydroxyphenyl ester (CAS No. 143121-08-8), 2,4-dichloro-5-(methoxycarbonyl)phenyl pyrrolidine-1-carboxylate (CAS No. 133636-94-9), 2,4-dichloro-5-[(propan-2-yloxy)carbonyl]phenyl pyrrolidine-1-carboxylate (CAS No. 133636-96-1), 1-piperidinecarboxylic acid, 2,4-dichloro-5-(2-propynyloxy)phenyl ester (CAS No. 87374-78-5), 2,4-dichloro-5-(prop-2-yn-1-yloxy)phenyl pyrrolidine-1-carboxylate (CAS No. 87365-63-7), 2,4-dichloro-5-(prop-2-yn-1-yloxy)phenyl 4,4-difluoropiperidine-1-carboxylate (CAS No. 138926-22-4), 1-pyrrolidinecarboxylic acid, 3,3-difluoro-2,4-dichloro-5-(2-propyn-1-yloxy)phenyl ester (CAS No. 143121-10-2), 4-chloro-2-fluoro-5-[(propan-2-yloxy)carbonyl]phenyl pyrrolidine-1-carboxylate (CAS No. 133636-98-3), etc.

The thiadiazoles herbicide includes fluthiacet and thidiazimin, but not limited thereto.

The oxadiazoles herbicide includes oxadiargyl and oxadiazon, but not limited thereto.

The triazolinones herbicide includes carfentrazone, sulfentrazone and azafenidin, but not limited thereto.

The oxazolidinediones herbicide includes pentoxazone, but not limited thereto.

The other herbicide includes pyraclonil, flufenpyr-ethyl and proflumizox, but not limited thereto.

The herbicide-tolerant PPO gene provided herein may be introduced into a plant or an alga by various methods known in the art, and preferably, by using an expression vector for plant or alga transformation.

In case of plant transformation, an appropriate promoter which may be included in the vector may be any promoter generally used in the art for introduction of the gene into the plant. For example, the promoter may include an SP6 promoter, a T7 promoter, a T3 promoter, a PM promoter, a maize ubiquitin promoter, a cauliflower mosaic virus (CaMV) 35S promoter, a nopaline synthase (nos) promoter, a figwort mosaic virus 35S promoter, a sugarcane bacilliform virus promoter, a commelina yellow mottle virus promoter, a light-inducible promoter from the small subunit of ribulose-1,5-bisphosphate carboxylase (ssRUBISCO), a rice cytosolic triosephosphate

isomerase (TPI) promoter, an adenine phosphoribosyltransferase (APRT) promoter of *A. thaliana*, an octopine synthase promoter, and a BCB (blue copper binding protein) promoter, but is not limited thereto.

Further, the vector may include a poly A signal sequence causing polyadenylation of 3'-terminus, and for example, it may include NOS 3'-end derived from a nopaline synthase gene of *Agrobacterium tumefaciens*, an octopine synthase terminator derived from an octopine synthase gene of *Agrobacterium tumefaciens*, 3'-end of protease inhibitor I or II gene of tomato or potato, a CaMV 35S terminator, a rice α -amylase terminator RAmy1 A, and a phaseoline terminator, but is not limited thereto.

In addition, chloroplast-specific promoter, nucleus promoter, constitutive promoter, or inducible promoter may be used for introduction of the gene into the algae as a promoter. The herbicide-tolerant PPO gene or its variant provided herein may be designed in order to operationally link to 5' UTR or 3' UTR, thereby expressing function in nucleus of algae. In addition, the vector may further comprise a transcriptional regulatory sequence which is appropriate to transformation of algae. A recombinant gene conferring herbicide tolerance may be integrated to genome of nucleus or genome of chloroplast in a host alga, but not limited thereto.

In addition, in the vector, a transit peptide required for targeting to chloroplasts may be linked to 5'-end of the PPO gene in order to express the herbicide-tolerant PPO gene in the chloroplasts.

In addition, optionally, the vector may further include a gene encoding selectable marker as a reporter molecule, and example of the selectable marker may include antibiotics (e.g., neomycin, carbenicillin, kanamycin, spectinomycin, hygromycin, bleomycin, chloramphenicol, ampicillin etc.) or herbicide (glyphosate, glufosinate, phosphinothricin, etc.)-tolerant genes, but is not limited thereto.

Further, the recombinant vector for plant expression may include an *Agrobacterium* binary vector, a cointegration vector, or a general vector which has no T-DNA region but is designed to be expressed in the plant. Of them, the binary vector refers to a vector containing two separate vector systems harboring one plasmid responsible for migration consisting of left border (LB) and right border (RB) in Ti

(tumor inducible) plasmid, and the other plasmid for target gene-transferring, and the vector may include a promoter region and a polyadenylation signal sequence for expression in plants.

When the binary vector or cointegration vector is used, a strain for transformation of the recombinant vector into the plant is preferably *Agrobacterium* (5 *Agrobacterium*-mediated transformation). In this regard, *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* may be used. In addition, when the vector having no T-DNA region is used, electroporation, particle bombardment, polyethylene glycol-mediated uptake, etc. may be used for introduction of the recombinant plasmid into the 10 plant.

The plant transformed with the gene by the above method may be re-differentiated into a plant through callus induction, rhizogenesis, and soil acclimatization using a standard technique known in the art.

The plant subjected to transformation herein is understood by a meaning 15 including a plant cell (containing a suspension-cultured cell), a protoplast, a callus, a hypocotyl, a seed, a cotyledon, a shoot as well as a mature plant.

Further, the scope of the transformant includes a transformant introduced with the gene as well as a clone or progeny thereof (T_1 generation, T_2 generation, T_3 generation, T_4 generation, T_5 generation, or any subsequent generations). For example, 20 the transformed plant also includes a plant having the inherited herbicide tolerance traits as sexual and asexual progeny of the plant transformed with the gene provided herein. The scope of the present invention also includes all mutants and variants showing the characteristics of the initial transformed plant, together with all hybridization and fusion products of the plant transformed with the gene provided herein. Furthermore, the 25 scope of the present invention also includes a part of the plant, such as a seed, a flower, a stem, a fruit, a leaf, a root, a tuber, and/or a tuberous root, which is originated from a transformed plant which is transformed in advance by the method of the present invention, or a progeny thereof, and is composed of at least a part of the transformed cells.

30 The plant, to which the present invention is applied, is not particularly limited

to, but may be at least one selected from the group consisting of monocotyledonous or dicotyledonous plants. Further, the plant includes herbaceous plants or woody plants. The monocotyledonous plant may include plants belonging to the family Alismataceae, Hydrocharitaceae, Juncaginaceae, Scheuchzeriaceae, Potamogetonaceae, Najadaceae, 5 Zosteraceae, Liliaceae, Haemodoraceae, Agavaceae, Amaryllidaceae, Dioscoreaceae, Pontederiaceae, Iridaceae, Burmanniaceae, Juncaceae, Commelinaceae, Eriocaulaceae, Gramineae (Poaceae), Araceae, Lemnaceae, Sparganiaceae, Typhaceae, Cyperaceae, Musaceae, Zingiberaceae, Cannaceae, Orchidaceae, but not limited thereto.

The dicotyledonous plant may include plants belonging to the family 10 Diapensiaceae, Clethraceae, Pyrolaceae, Ericaceae, Myrsinaceae, Primulaceae, Plumbaginaceae, Ebenaceae, Styracaceae, Symplocaceae, Symplocaceae, Oleaceae, Loganiaceae, Gentianaceae, Menyanthaceae, Apocynaceae, Asclepiadaceae, Rubiaceae, Polemoniaceae, Convolvulaceae, Boraginaceae, Verbenaceae, Labiatae, Solanaceae, Scrophulariaceae, Bignoniaceae, Acanthaceae, Pedaliaceae, Orobanchaceae, 15 Gesneriaceae, Lentibulariaceae, Phrymaceae, Plantaginaceae, Caprifoliaceae, Adoxaceae, Valerianaceae, Dipsacaceae, Campanulaceae, Compositae, Myricaceae, Juglandaceae, Salicaceae, Betulaceae, Fagaceae, Ulmaceae, Moraceae, Urticaceae, Santalaceae, Loranthaceae, Polygonaceae, Phytolaccaceae, Nyctaginaceae, Aizoaceae, Portulacaceae, Caryophyllaceae, Chenopodiaceae, Amaranthaceae, Cactaceae, 20 Magnoliaceae, Illiciaceae, Lauraceae, Cercidiphyllaceae, Ranunculaceae, Berberidaceae, Lardizabalaceae, Menispermaceae, Nymphaeaceae, Ceratophyllaceae, Cabombaceae, Saururaceae, Piperaceae, Chloranthaceae, Aristolochiaceae, Actinidiaceae, Theaceae, Guttiferae, Droseraceae, Papaveraceae, Capparidaceae, Cruciferae, Platanaceae, Hamamelidaceae, Crassulaceae, Saxifragaceae, Eucommiaceae, Pittosporaceae, 25 Rosaceae, Leguminosae, Oxalidaceae, Geraniaceae, Tropaeolaceae, Zygophyllaceae, Linaceae, Euphorbiaceae, Callitrichaceae, Rutaceae, Simaroubaceae, Meliaceae, Polygalaceae, Anacardiaceae, Aceraceae, Sapindaceae, Hippocastanaceae, Sabiaceae, Balsaminaceae, Aquifoliaceae, Celastraceae, Staphyleaceae, Buxaceae, Empetraceae, Rhamnaceae, Vitaceae, Elaeocarpaceae, Tiliaceae, Malvaceae, Sterculiaceae, 30 Thymelaeaceae, Elaeagnaceae, Flacourtiaceae, Violaceae, Passifloraceae, Tamaricaceae,

Elatinaceae, Begoniaceae, Cucurbitaceae, Lythraceae, Punicaceae, Onagraceae, Haloragaceae, Alangiaceae, Cornaceae, Araliaceae, and Umbelliferae(Apiaceae), but not limited thereto.

In a specific embodiment, the plant may be one or more kinds selected from the group consisting of food crops such as rice, wheat, barley, corn, soybean, potato, red bean, oat, and sorghum; vegetable crops such as Chinese cabbage, radish, red pepper, strawberry, tomato, watermelon, cucumber, cabbage, oriental melon, pumpkin, welsh anion, anion, and carrot; crops for special use such as ginseng, tobacco, cotton, soilage, forage, sesame, sugar cane, sugar beet, *Perilla* sp., peanut, rapeseed, grass, and castor-oil plant; fruit trees such as apple tree, pear tree, jujube tree, peach tree, kiwi fruit tree, grape tree, citrus fruit tree, persimmon tree, plum tree, apricot tree and banana tree; woody plants such as pine, palm oil, and eucalyptus; flowering crops such as rose, gladiolus, gerbera, carnation, chrysanthemum, lily and tulip; and fodder crops such as ryegrass, red clover, orchardgrass, alfalfa, tall fescue and perennial ryegrass, but is not limited thereto. As a specific embodiment, the plant may be one or more kinds selected from the group consisting of dicotyledonous plants such as arabidopsis, potato, eggplant, tobacco, red pepper, tomato, burdock, crown daisy, lettuce, balloon flower, spinach, chard, sweet potato, celery, carrot, water dropwort, parsley, Chinese cabbage, cabbage, radish, watermelon, oriental melon, cucumber, pumpkin, gourd, strawberry, soybean, mung bean, kidney bean, and pea; and monocotyledonous plants such as rice, wheat, barley, corn, sorghum, etc., but are not limited thereto.

The alga, to which the present invention is applied, is not particularly limited to, but includes Prokaryotic algae or Eukaryotic algae. For example, the alga may be cyanobacteria, green algae, red algae, brown algae, macroalgae, or microalgae.

The cyanobacteria includes Chroococcales phylum (for example, *Aphanocapsa*, *Aphanothece*, *Chamaesiphon*, *Chondrocystis*, *Chroococcus*, *Chroogloeocystis*, *Crocospaera*, *Cyanobacterium*, *Cyanobium*, *Cyanodictyon*, *Cyanosarcina*, *Cyanothece*, *Dactylococcopsis*, *Gloeocapsa*, *Gloeothece*, *Halothece*, *Johannesbaptistia*, *Merismopedia*, *Microcystis*, *Radiocystis*, *Rhabdoderma*, *Snowella*, *Synechococcus*, *Synechocystis*, *Thermosynechococcus*, *Woronichinia*), *Gloeobacteria* phylum,

Nostocales phylum (for example, Microchaetaceae, Nostocaceae, Rivulariaceae, Scytonemataceae), Oscillatoriales phylum (for example, Arthronema, Arthrospira, Blennothrix, Crinalium, Geitlerinema, Halomicronema, Halospirulina, Hydrocoleum, Jaaginema, Katagnymene, Komvophoron, Leptolyngbya, Limnothrix, Lyngbya, 5 Microcoleus, Oscillatoria, Phormidium, Planktothricoides, Planktothrix, Plectonema, Pseudanabaena, Pseudophormidium, Schizothrix, Spirulina, Starria, Symploca, Trichodesmium, Tychonema), Pleurocapsales phylum (for example, Chroococcidiopsis, Dermocarpa, Dermocarpella, Myxosarcina, Pleurocapsa, Solentia, Stanieria, Xenococcus), Prochlorales phylum, or Stigonematales phylum (for example, Capsosira, 10 Chlorogloeopsis, Fischerella, Hapalosiphon, Mastigocladopsis, Mastigocladus, Nostochopsis, Stigonema, Symphyonema, Symphonemopsis, Umezakia, Westiellopsis), etc.

As another example of algae, Chlorophyta, Chlamydomonas, Volvocales, Dunaliella, Scenedesmus, Chlorella, or Hematococcm may be exemplified.

15 As other example of algae, Phaeodactylum tricornutum, Amphiprora hyaline, Amphora spp., Chaetoceros muelleri, Navicula saprophila, Nitzschia communis, Scenedesmus dimorphus, Scenedesmus obliquus, Tetraselmis suecica, Chlamydomonas reinhardtii, Chlorella vulgaris, Haematococcus pluvialis, Neochloris oleoabundans, Synechococcus elongatus, Botryococcus braunii, Gloeobacter violaceus, Synechocystis, 20 Thermosynechococcus elongatus, Nannochloropsis oculata, Nannochloropsis salina, Nannochloropsis gaditana, Isochrysis galbana, Botryococcus sudeticus, Euglena gracilis, Neochloris oleoabundans, Nitzschia palea, Pleurochrysis carterae, Tetraselmis chuii, Pavlova spp., Aphanocapsa spp., Synechosystis spp., Nannochloris spp., etc. may be exemplified. However, it is not limited to kinds listed above, and algae belonging to 25 other various genus and family may be comprised.

The plant or alga introduced with the herbicide-tolerant PPO or its variant provided herein may exhibit tolerance against two or more of PPO-inhibiting herbicides.

Therefore, the technology provided herein may be used to control weeds or remove undesired aquatic organisms by using two or more kinds of PPO-inhibiting 30 herbicides sequentially or simultaneously.

One embodiment provides a method of controlling weeds in a cropland, comprising a step of providing the cropland with a plant comprising the herbicide-tolerant PPO protein, its variant, or a gene encoding thereof described above, and a step of applying an effective dosage of protoporphyrinogen oxidase-inhibiting herbicide to the cropland.

Another embodiment provides a method of removing an undesired aquatic organism from a culture medium, comprising a step of providing a culture medium with algae comprising the herbicide-tolerant PPO protein, its variant, or a gene encoding thereof described above, and a step of applying an effective dosage of protoporphyrinogen oxidase-inhibiting herbicide to the culture medium.

In addition, the herbicide-tolerant PPO protein, its variant, or a gene encoding thereof provided herein may be used in combination of a second herbicide-tolerant polypeptide or a gene encoding thereof.

Therefore, the plant or alga introduced with the herbicide-tolerant PPO provided herein may exhibit tolerance against two or more of herbicides which are different from each other in mechanism of action. In the present invention, two or more of different herbicides including the PPO-inhibiting herbicide, which are different from each other in mechanism of action, may be used sequentially or simultaneously, thereby controlling weeds and/or removing undesired aquatic organisms. Hereinafter, the herbicide which is different from the PPO-inhibiting herbicide in the mechanism of action is called "second herbicide".

One embodiment provides a composition for conferring or enhancing herbicide tolerance of plants or algae, comprising the above-described herbicide-tolerant PPO protein, its variant, or a gene encoding thereof; and the second herbicide-tolerant polypeptide or a gene encoding thereof.

Another embodiment provides a transformant having herbicide tolerance of plants or algae, or a clone or progeny thereof, comprising the above-described herbicide-tolerant PPO protein, its variant, or a gene encoding thereof; and the second herbicide-tolerant polypeptide or a gene encoding thereof.

Other embodiment provides a method of preparing plants or algae having

herbicide tolerance, comprising a step of transforming algae, or plant cell, protoplast, callus, hypocotyl, seed, cotyledon, shoot, or whole plant with the above-described herbicide-tolerant PPO protein, its variant, or a gene encoding thereof; and the second herbicide-tolerant polypeptide or a gene encoding thereof.

5 Other embodiment provides a method of controlling weeds in a cropland, comprising a step of providing the cropland with a plant comprising the above-described herbicide-tolerant PPO protein, its variant, or a gene encoding thereof; and the second herbicide-tolerant polypeptide or a gene encoding thereof, and a step of applying an effective dosage of protoporphyrinogen oxidase-inhibiting herbicide to the cropland.

10 Other embodiment provides a method of removing an undesired aquatic organism from a culture medium, comprising a step of providing a culture medium with algae comprising the herbicide-tolerant PPO protein, its variant, or a gene encoding thereof; and the second herbicide-tolerant polypeptide or a gene encoding thereof, and a step of applying an effective dosage of protoporphyrinogen oxidase-inhibiting herbicide
15 to the culture medium.

For example, the plant or alga further includes the second herbicide- tolerance polypeptide or a gene encoding thereof, thereby having novel and/or enhanced tolerance against the second herbicide.

For example, the second herbicide may include cell division-inhibiting
20 herbicides, photosynthesis-inhibiting herbicides, amino acid synthesis-inhibiting herbicides, plastid-inhibiting herbicides, cell membrane-inhibiting herbicides, and/or any combinations thereof, but is not limited thereto. The second herbicide may be exemplified by glyphosate, glufosinate, dicamba, 2,4-D (2,4-dichlorophenoxyacetic acid), ALS (acetolactate synthase)-inhibiting herbicides (for example, imidazolidinone,
25 sulfonylurea, triazole pyrimidine, sulphonanilide, pyrimidine thiobenzoate, etc.), photosystem II-inhibiting herbicides, phenylurea-based herbicides, plastid-inhibiting herbicides, bromoxynil-based herbicides, and/or any combinations thereof, but is not limited thereto.

For example, the second herbicide-tolerant polypeptide may be exemplified as
30 one or more kinds selected from the group consisting of glyphosate herbicide-tolerant

EPSPS (glyphosate tolerant 5-enolpyruvylshikimate-3-phosphate synthase), GOX (glyphosate oxidase), GAT (glyphosate-N-acetyltransferase) or glyphosate decarboxylase; glufosinate herbicide-tolerant PAT (phosphinothricin-N-acetyltransferase); dicamba herbicide-tolerant DMO (dicamba monooxygenase); 2,4-D
 5 herbicide-tolerant 2,4-D monooxygenase or AAD (aryloxyalkanoate dioxygenase); ALS-inhibiting sulfonylurea-based herbicide-tolerant ALS (acetolactate synthase), AHAS (acetohydroxyacid synthase), or AtAHASL (acetohydroxyacid synthase large subunit); photosystem II-inhibiting herbicide-tolerant photosystem II protein D1; phenylurea-based herbicide-tolerant cytochrome P450; plastid-inhibiting herbicide-
 10 tolerant HPPD (hydroxylphenylpyruvate dioxygenase); bromoxynil herbicide-tolerant nitrilase; and any combinations thereof, but is not limited thereto.

Further, the gene encoding the second herbicide-tolerant polypeptide may be exemplified as one or more kinds selected from the group consisting of glyphosate herbicide-tolerant cp4 epsps, epsps (AG), mepsps, 2mepsps, goxv247, gat4601 or
 15 gat4621 gene; glufosinate herbicide-tolerant bar, pat or pat (SYN) gene; dicamba herbicide-tolerant dmo gene; 2,4-D herbicide-tolerant AAD-1 or AAD-12 gene; ALS-inhibiting sulfonylurea-based herbicide-tolerant ALS, GM-HRA, S4-HRA, ZM-HRA, Csr1, Csr1-1, Csr1-2, SurA or SurB; photosystem II-inhibiting herbicide-tolerant psba gene; phenylurea herbicide-tolerant CYP76B1 gene; isoxaflutole herbicide-tolerant
 20 HPPDPF W336 gene; bromoxynil herbicide-tolerant bxn gene; and any combinations thereof, but is not limited thereto.

【Advantageous Effects】

A variant of herbicide-tolerant PPO protein or a gene encoding thereof provided
 25 herein is applied to plants or algae, thereby conferring and/or enhancing more excellent herbicide tolerance traits, and the selective control is performed using herbicides, thereby economically controlling weeds or removing aquatic organisms.

【Description of Drawings】

30 FIG. 1 is a photograph showing cell growth level in case of treating tiafenacil at

a concentration of 0 μ M (micromole), 25 μ M, 50 μ M, 100 μ M and 400 μ M, after transforming PPO-deficient BT3 *E. coli* (BT3(Δ PPO)) with pACBB empty vector (indicated by V), *A. thaliana* PPO1 gene (indicated by WT), CyPPO2 wild type gene (indicated by Cy2), CyPPO4 wild type gene (indicated by Cy4), or CyPPO8 wild type gene (indicated by Cy8).

FIG. 2 is the map of pET29b vector.

FIG. 3 is the map of pACBB-eGFP vector.

FIG. 4 is the map of pET303-CT-His vector.

FIG. 5 is a photograph showing cell growth level of PPO-deficient BT3 *E. coli* (BT3(Δ PPO)) transformant transformed with CyPPO2 wild type gene (indicated by Cy2 WT), or various CyPPO2 mutant genes, when treated with tiafenacil at a concentration of 0 μ M, 5 μ M, 25 μ M, 50 μ M, 100 μ M and 200 μ M, respectively.

FIG. 6 is a photograph showing cell growth level of BT3(Δ PPO) transformant transformed with Cy2 WT, or various CyPPO2 mutant genes, when treated with saflufenacil at a concentration of 0 μ M, 5 μ M, 25 μ M, 50 μ M, 100 μ M and 200 μ M, respectively.

FIG. 7 is a photograph showing cell growth level of BT3(Δ PPO) transformant transformed with Cy2 WT or various CyPPO2 mutant genes, when treated with flumioxazin at a concentration of 0 μ M, 5 μ M, 25 μ M, 50 μ M, 100 μ M and 200 μ M, respectively.

FIG. 8 is a photograph showing cell growth level of BT3(Δ PPO) transformant transformed with Cy2 WT or various CyPPO2 mutant genes, when treated with fomesafen at a concentration of 0 μ M, 5 μ M, 25 μ M, 50 μ M, 100 μ M and 200 μ M, respectively.

FIG. 9 is a photograph showing cell growth level of BT3(Δ PPO) transformant transformed with Cy2 WT or various CyPPO2 mutant genes, when treated with acifluorfen at a concentration of 0 μ M, 5 μ M, 25 μ M, 50 μ M, 100 μ M and 200 μ M, respectively.

FIG. 10 is a photograph showing cell growth level of BT3(Δ PPO) transformant transformed with Cy2 WT or various CyPPO2 mutant genes, when treated with

pyraclonil at a concentration of 0 μ M, 5 μ M, 25 μ M, 50 μ M, 100 μ M and 200 μ M, respectively.

FIG. 11 is a photograph showing cell growth level of BT3(Δ PPO) transformant transformed with Cy2 WT or various CyPPO2 mutant genes, when treated with sulfentrazone at a concentration of 0 μ M, 5 μ M, 25 μ M, 50 μ M, 100 μ M and 200 μ M, respectively.

FIG. 12 is a photograph showing cell growth level of BT3(Δ PPO) transformant transformed with Cy2 WT or various CyPPO2 mutant genes, when treated with pentoxazone at a concentration of 0 μ M, 5 μ M, 25 μ M, 50 μ M, 100 μ M and 200 μ M, respectively.

FIG. 13 is a photograph showing cell growth level of BT3(Δ PPO) transformant transformed with Cy2 WT or various CyPPO2 mutant genes, when treated with pyraflufen-ethyl at a concentration of 0 μ M, 5 μ M, 25 μ M, 50 μ M, 100 μ M and 200 μ M, respectively.

FIG. 14 is a photograph showing cell growth level of BT3(Δ PPO) transformant transformed with CyPPO4 wild type gene (indicated by Cy4 WT) or various CyPPO4 mutant genes, when treated with tiafenacil at a concentration of 0 μ M, 5 μ M, 25 μ M, 50 μ M, 100 μ M and 200 μ M, respectively.

FIG. 15 is a photograph showing cell growth level of BT3(Δ PPO) transformant transformed with Cy4 WT or various CyPPO4 mutant genes, when treated with saflufenacil at a concentration of 0 μ M, 5 μ M, 100 μ M and 200 μ M, respectively.

FIG. 16 is a photograph showing cell growth level of BT3(Δ PPO) transformant transformed with Cy4 WT or various CyPPO4 mutant genes, when treated with flumioxazin at a concentration of 0 μ M, 5 μ M, 100 μ M and 200 μ M, respectively.

FIG. 17 is a photograph showing cell growth level of BT3(Δ PPO) transformant transformed with CyPPO8 wild type gene (indicated by Cy8 WT) or various CyPPO8 mutant genes, when treated with tiafenacil at a concentration of 0 μ M, 5 μ M, 25 μ M, 50 μ M, 100 μ M, 200 μ M and 400 μ M, respectively, wherein the top is a result obtained by using pET303-CT-His vector, and the bottom is a result obtained by using pACBB vector.

FIG. 18 is a photograph showing cell growth level of BT3(Δ PPO) transformant transformed with Cy8 WT or various CyPPO8 mutant genes, when treated with saflufenacil at a concentration of 0 μ M, 5 μ M, 25 μ M, 50 μ M, 100 μ M and 200 μ M, respectively.

5 FIG. 19 is a photograph showing cell growth level of BT3(Δ PPO) transformant transformed with Cy8 WT or various CyPPO8 mutant genes, when treated with fomesafen at a concentration of 0 μ M, 5 μ M, 25 μ M, 50 μ M, 100 μ M and 200 μ M, respectively.

FIG. 20 is a photograph showing cell growth level of BT3(Δ PPO) transformant
10 transformed with Cy8 WT or various CyPPO8 mutant genes, when treated with acifluorfen at a concentration of 0 μ M, 5 μ M, 25 μ M, 50 μ M, 100 μ M and 200 μ M, respectively.

FIG. 21 is a photograph showing cell growth level of BT3(Δ PPO) transformant transformed with Cy8 WT or various CyPPO8 mutant genes, when treated with
15 flumioxazin at a concentration of 0 μ M, 5 μ M, 25 μ M, 50 μ M, 100 μ M and 200 μ M, respectively.

FIG. 22 is a photograph showing cell growth level of BT3(Δ PPO) transformant transformed with Cy8 WT or various CyPPO8 mutant genes, when treated with sulfentrazone at a concentration of 0 μ M, 5 μ M, 25 μ M, 50 μ M, 100 μ M and 200 μ M,
20 respectively.

FIG. 23 is a photograph showing cell growth level of BT3(Δ PPO) transformant transformed with Cy8 WT or various CyPPO8 mutant genes, when treated with pentoxazone at a concentration of 0 μ M, 5 μ M, 25 μ M, 50 μ M, 100 μ M and 200 μ M, after respectively.

25 FIG. 24 is a photograph showing cell growth level of BT3(Δ PPO) transformant transformed with Cy8 WT or various CyPPO8 mutant genes, when treated with pyraflufen-ethyl at a concentration of 0 μ M, 5 μ M, 25 μ M, 50 μ M, 100 μ M and 200 μ M, respectively.

FIG. 25 is a photograph showing cell growth level of BT3(Δ PPO) transformant
30 transformed with Cy8 WT or various CyPPO8 mutant genes, when treated with

pyraclonil at a concentration of 0 μ M, 5 μ M, 25 μ M, 50 μ M, 100 μ M and 200 μ M, respectively.

FIG. 26 is a schematic diagram showing a recombinant vector for preparing a fusion protein wherein MBP (maltose binding protein) and PPO protein are fused.

5 FIG. 27 is the map of pMAL-c2X vector.

FIG. 28 shows the result of SDS-PAGE to isolate and purify CyPPO2 wild type protein and CyPPO2-Y373M variant protein.

FIG. 29 shows the result of SDS-PAGE to isolate and purify CyPPO4 wild type protein and CyPPO4-Y375M variant protein.

10 FIG. 30 shows the result of SDS-PAGE to isolate and purify CyPPO8 wild type protein and CyPPO8-F363M variant protein.

FIG. 31 is a schematic diagram exemplarily showing the structure of binary vector for plant transformation of CyPPO genes.

15 FIG. 32 shows germination level of seeds of *A. thaliana* (T₂) transformant wherein mutant gene of CyPPO2, CyPPO4 or CyPPO8 is introduced, when germinating the seeds in a medium comprising various concentrations of tiafenacil. Col-O means non-transgenic *A. thaliana*.

FIG. 33 shows the western blot results showing expression level of CyPPO variant proteins in *A. thaliana* (T₂) transfected with mutant gene of CyPPO2, CyPPO4
20 or CyPPO8. Col-0 means non-transgenic *A. thaliana*.

FIG. 34 shows a result observed at the 7 day after spraying 5 μ M of tiafenacil to the transformed *A. thaliana* (T₃) wherein gene encoding CyPPO2 Y373M variant or CyPPO8 F363M variant is introduced. Col-O means non-transgenic *A. thaliana*.

25 FIG. 35 shows a result observed at the 7 day after spraying 5 μ M of saflufenacil to transformed *A. thaliana* (T₃) wherein gene encoding CyPPO2 Y373M variant, CyPPO4 Y375M variant or CyPPO8 F363M variant is introduced. Col-O means non-transgenic *A. thaliana*.

FIG. 36 shows a result observed at the 7 day after spraying 5 μ M of Fomesafen to transformed *A. thaliana* (T₃) wherein gene encoding CyPPO2 Y373M variant or
30 CyPPO8 F363M variant is introduced. Col-O means non-transgenic *A. thaliana*.

FIG. 37 shows a result observed at the 7 day after spraying 25 μ M or 5 μ M of tiafenacil or 75 μ M of saflufenacil to transformed *A. thaliana* (T₃ or T₂) wherein gene encoding Y373I, Y373L, Y373V, Y373C, Y373M, V318M+Y373I, V173S+A175C+Y373M, or A175C+V318M+Y373M variant of CyPPO2 is introduced
5 respectively.

FIG. 38 shows a result observed at the 7 day after spraying 25 μ M or 10 μ M of tiafenacil or 100 μ M concentration of saflufenacil to transformed *A. thaliana* (T₃ or T₂) wherein gene encoding F363V, F363L, A162L, or A162C+V308M+F363M variant of CyPPO8 is introduced.

10 FIG. 39 is the map of pCAMBIA3301 vector.

FIG. 40 shows a result observed at the 7 day after spraying PPO-inhibiting herbicide to wild type Dongjin rice and T₀ generation of transformants of Dongjin rice transformed with CyPPO2 Y373M mutant gene or CyPP08 F363M mutant gene, wherein A is an image after 853g ai/ha tiafenacil treatment, and B is an image after
15 2,087g ai/ha saflufenacil treatment.

FIG. 41 shows a result observed at the 7 day image after spraying PPO-inhibiting herbicide to CyPPO2 Y373M T₂ transformant line no. 3 and CyPPO8 F363M T₂ transformant line no. 3 of Dongjin rice, wherein A is an image after 420g ai/ha tiafenacil treatment, and B is an image after 840g ai/ha saflufenacil treatment (1: young
20 leaf → 4: old leaf). Dongjin means non transgenic rice (cultivar).

FIG. 42 is the western blot result showing expression of CyPPO2 Y373M variant protein in the leaves of CyPPO2 Y373M transformant of Dongjin rice.

FIG. 43 is the western blot result showing expression of CyPPO8 F363M variant protein in the leaves of CyPPO8 F363M transformant of Dongjin rice.

25 FIG. 44 is the southern blotting result examining whether CyPPO8 F363M is present in CyPPO8 F363M transformant of Dongjin rice.

FIG. 45 is a photograph showing tolerance level against various herbicides of *A. thaliana* transformants (T₃) wherein Y373I of CyPPO2 and F363V of CyPPO8 mutant genes are respectively introduced.

30 FIG. 46 is a photograph showing herbicide tolerance level of T₄ generation of *A.*

thaliana transformants wherein Y373I of CyPPO2 and F363V of CyPPO8 mutant genes are respectively introduced.

FIG. 47 is a photograph showing herbicide tolerance level of T₅ generation of CyPPO2 Y373I-introduced transformant and CyPPO8 F363V-introduced transformant of *A. thaliana*.

FIG. 48 is the western blot result showing PPO protein expression of T₄ generation of CyPPO2 Y373I-introduced transformant and CyPPO8 F363V-introduced transformant of *A. thaliana*.

FIG. 49 is the western blot result showing PPO protein expression of T₅ generation of CyPPO2 Y373I-introduced transformant and CyPPO8 F363V-introduced transformant of *A. thaliana*.

FIG. 50 is a structural diagram exemplarily showing the structure of vector for soybean transformation.

FIG. 51 is the southern blotting result examining whether mutant gene is present in CyPP02 Y373M transformant and CyPPO8 F363M transformant of soybean.

FIG. 52 is a photograph showing herbicide tolerance level of T₂ generation of CyPP02 Y373M transformant and CyPPO8 F363M transformant of soybean. Kwangan means non-transgenic soybean (cultivar)

FIG. 53 is the western blot result examining whether CyPPO2 Y373M protein and CyPPO8 F363M protein are expressed in the leaves of CyPPO2 Y373M transformant and CyPPO8 F363M transformant of soybean, respectively.

FIG. 54 is a photograph showing growth image before (upper) and after (bottom) treating tiafenacil to CyPPO8 F363M transformant of rapeseed. Youngsan means non-transgenic rapeseed (cultivar).

FIG. 55 is a photograph showing growth image before (upper) and after (bottom) treating saflufenacil to CyPPO8 F363M transformant of rapeseed. Wild type means non-transgenic rapeseed (cultivar).

FIG. 56 shows the western blot result examining whether CyPPO8 F363M protein is expressed in the leaves of CyPPO8 F363M transformant of rapeseed (upper) and showing the Coomassie stain result (bottom).

【Mode for Invention】

Hereinafter, the present invention will be described in detail with reference to Examples. However, these Examples are for illustrative purposes only, and the invention is not intended to be limited by these Examples.

Example 1. Isolation of PPO gene from prokaryote

Oscillatoria nigro-viridis PCC 7112, *Lyngbya* sp. PCC 8106 strain, and *Halotheca* sp. PCC 7418 strain were provided by the Institut Pasteur (France), and PPO genes were isolated from each strain. Each PPO gene was synthesized using codon-optimized sequence information for efficient herbicide resistance screening in BT3 *E. coli*. The synthesized PPO genes were amplified using the primers of Table 1.

One microliter (1 μ l) of each template (genomic DNA of each strain), 5 μ l of 10X buffer, 1 μ l of dNTP mixture (each 10 mM), 1 μ l of a forward primer (refer to Table 1; 10 μ M), 1 μ l of a reverse primer (refer to Table 1; 10 μ M), 40 μ l of DDW, and 1 μ l of Pfu-X (Solgent, 2.5 unit/ μ l) were mixed to prepare 50 μ l of PCR reaction mixture, and amplification was performed under conditions of at 94 °C for 4 minutes, and 25 cycles (at 94 °C for 30 seconds, at 56 °C for 30 seconds and at 72 °C for 1.5 minutes), at 72 °C for 5 minutes.

PPO isolated from *Oscillatoria nigro-viridis* PCC 7112 was designated as CyPPO2, PPO isolated from *Lyngbya* sp. PCC 8106 strain was designated as CyPPO4, and PPO isolated from *Halotheca* sp. PCC 7418 strain was designated as CyPPO8.

Further, respective nucleotide sequences of CyPPO2, CyPPO4, and CyPPO8 and amino acid sequences encoded by the corresponding sequences were examined, and represented by SEQ ID NOS: 1 to 6.

【Table 1】

Strain	Primer	Sequence	SEQ ID NO:
<i>Oscillatoria nigro-viridis</i>	CyPPO2_F	CCCCGGATCCATGGAAGTTCTTGATACTCT	12
	CyPPO2_R	CCCCCTCGAGGATTGACC TGGTATCACTCT	13

PCC 7112			
<i>Lyngbya</i> sp.	CyPPO4_F	CCCCGGATCCATGACCCATGTTTTGGATTC	14
PCC 8106	CyPPO4_R	CCCCCTCGAGCTGTCCTAAAAATGATAAAATCTCG	15
<i>Halotheca</i> sp.	CyPPO8_F	CCCCGGATCCATGATAGATACTCTTATAGTGGG	16
PCC 7418	CyPPO8_R	CCCCCTCGAGTCCTAAGTAATCTAAACTG	17

The herbicide tolerance of CyPPO2, CyPPO4, and CyPPO8 prepared above was tested using PPO-deficient *E. coli* (BT3 (Δ PPO)). BT3 (Δ PPO) strain was provided by Hokkaido University (Japan) and it is an *E. coli* strain which is deficient in hemG-type PPO and has Kanamycin tolerance (refer to "Watanabe et al., Dual targeting of spinach protoporphyrinogen oxidase II to mitochondria and chloroplasts by alternative use of two in-frame inhibition codons, JBC 2001 276(23):20474–20481; Che et al., Molecular Characterization and Subcellular Localization of Protoporphyrinogen Oxidase in Spinach Chloroplasts, Plant Physiol. 2000 Sep;124(1):59-70").

BT3 (Δ PPO) was transformed with each CyPPO gene, and cultured on the LB (Luria-Bertani) agar media containing PPO-inhibiting herbicide, thereby examining whether the growth of the transformed *E. coli* was inhibited.

The specific test process was as follows:

CyPPO2, CyPPO4, and CyPPO8 genes prepared above are cloned in pACBB vector (Plasmid #32551; Addgene; refer to FIG. 3). The cloned plasmids were added to BT3 competent cell respectively, thereby transforming by a heat shock method. The transformed *E. coli* with each CyPPO gene was cultured in LB agar media containing chloramphenicol (34 μ g/ml, Duchefa).

Single colony of *E. coli* transformed with each CyPPO gene was cultured in 3 ml of LB broth containing chloramphenicol for overnight (220 rpm, 37 °C), and then the each culture was re-cultured with fresh media until absorbance (OD₆₀₀) became 0.5 to 1. It was diluted with LB broth to absorbance (OD₆₀₀) of 0.5. Again, the diluted solution was serially diluted 5 times by a factor of one tenth. Next, 10 μ l of each diluted solution was dropped on the LB agar media containing chloramphenicol (34 μ g/ml) and 0~400 μ M concentration of tiafenacil. The LB agar media were cultured at 37 °C under light condition, and level of growth inhibition was evaluated after 16 to 20 hours.

For comparison, the same test was conducted using BT3 *E. coli* transformant transformed with pACBB-eGFP vector (Plasmid #32551; Addgene; refer to FIG. 3) and BT3 *E. coli* transformant introduced with *A. thaliana*-derived wild type PPO gene (wild type AtPPO1) (SEQ ID NO: 8).

- 5 As shown in FIG. 1, the BT3 *E. coli* transformants of CyPPO2, CyPPO4, and CyPPO8 genes exhibited higher herbicide tolerance compared to *E. coli* transformants of AtPPO1 gene at all the concentrations of herbicide tested.

Example 2. Determination of PPO amino acid residues interacting with
PPO-inhibiting herbicides from PPO and PPO-inhibiting herbicide complex

In order to confirm the binding structure information of PPO protein and herbicide, tiafenacil was used as a representative example of PPO-inhibiting herbicides.

- In order to obtain a water-soluble CyPPO2 protein, 7 hydrophobic amino acid residues which were positioned in the thylakoid membrane binding domain in the amino acid of CyPPO2 (SEQ ID NO: 1) were substituted with hydrophilic residues. The gene encoding the amino acid residues-substituted variant protein of CyPPO2 was cloned to pET29b vector (Catalog Number: 69872-3; EMD Biosciences; refer to FIG. 2), and CyPPO2 protein was expressed using *E. coli* system. The expressed CyPPO2 protein was purified through nickel affinity chromatography and was crystallized. CyPPO2 and tiafenacil complex was obtained by soaking of 3 mM tiafenacil, and used for X-ray diffraction by synchrotron radiation accelerator. X-ray diffraction data of the 2.8 Å resolution of CyPPO2-tiafenacil complex crystals was obtained, and the three-dimensional structure was determined, and the binding position of tiafenacil in CyPPO2 protein was analyzed. As a result of structure analysis of CyPPO2 and tiafenacil complex, amino acids of S63, P91, R92, F169, V173, A175, E228, L229, P316, V318, F337, L340, G351, T352, I353 and Y373 positions of CyPPO2 protein (SEQ ID NO: 1) interacted with tiafenacil.

- And also, using the information derived from the structure of CyPPO2-tiafenacil complex, amino acid residues which interact with tiafenacil in CyPPO4 (SEQ ID NO: 3) and CyPPO8 (SEQ ID NO: 5) proteins (NCBI BLAST,

http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) were determined by sequence homology analysis as below.

The amino acids of CyPPO4 protein (SEQ ID NO: 3) interacting with tiafenacil are N63, S64, S66, P67, P92, R93, F170, S172, G173, V174, Y175, A176, R190, E230, L231, P318, V320, F339, G340, N341, L342, L352, G353, T354, I355, Y375, I424, V458, and R465. The amino acids of CyPPO8 protein (SEQ ID NO: 5) interacting with tiafenacil are P84, R85, F156, V160, A162, Q179, P306, V308, F327, L330, I343, N362, and F363.

10 **Example 3. Verification of PPO-inhibiting herbicide tolerance by PPO variants (test in *E. coli*)**

In order to enhance PPO-inhibiting herbicide tolerance of CyPPO2, CyPPO4 and CyPPO8, a mutation(s) at the position interacting with herbicide obtained in the Example 2 was introduced, respectively. BT3 (Δ PPO) was transformed with the mutated CyPPO gene, and cultured under the condition treated with PPO-inhibiting herbicide, thereby examining whether growth of transformed BT3 was inhibited. The wild type of each gene was used as a control.

The method of the experiment was as follows:

CyPPO2 and CyPPO4 cloned in pACBB vector (Plasmid #32551; Addgene; refer to FIG. 3) were mutated using primers of Tables 2 to 3, and CyPPO8 cloned in pACBB vector or pET303-CT-His vector (VT0163; Novagen; refer to FIG. 4) were mutated using primers of Table 4.

Fifty microliters of PCR reaction mixture was prepared by mixing 1 μ l of template, 5 μ l of 10X buffer, 1 μ l of dNTP mixture (each 10 mM), 1 μ l of a forward primer (10 μ M), 1 μ l of a reverse primer (10 μ M), 40 μ l of DDW, and 1 μ l of Pfu-X (Solgent, 2.5 unit/ μ l), and amplification was performed under conditions of at 94 °C for 4 minutes, and 17~25 cycles (at 94 °C for 30 seconds, at 56~65 °C for 30 seconds and at 72 °C for 3 minutes), at 72 °C for 5 minutes. 0.5 μ l of DpnI (New England Biolabs) was treated to each 5 μ l of PCR product, and incubated at 37 °C for 30 minutes. Reaction solution was transformed with *E. coli* by heat shock method. Each transformant was

cultured in LB agar media containing chloramphenicol or ampicillin. Each mutation was confirmed by plasmid DNA sequencing, and each plasmid was transformed to BT3 (Δ PPO).

【Table 2】

5 Primer for CyPPO2 mutation

No.	CyPPO2 mutation	Primer (Forward: top; Reverse: bottom) (5'→ 3')
1	Y373M	GACCTCTATGATTGGTGGAGCTACTGATAG (SEQ ID NO: 18)
		ACCAATCATAGAGGTCAATGTTTGCCATCC (SEQ ID NO: 19)
2	Y373V	GACCTCTGTGATTGGTGGAGCTACTGATAG (SEQ ID NO: 20)
		ACCAATCACAGAGGTCAATGTTTGCCATCC (SEQ ID NO: 21)
3	Y373I	GACCTCTATCATTGGTGGAGCTACTGATAG (SEQ ID NO: 22)
		ACCAATGATAGAGGTCAATGTTTGCCATCC (SEQ ID NO: 23)
4	Y373T	GACCTCTACAATTGGTGGAGCTACTGATAG (SEQ ID NO: 24)
		ACCAATTGTAGAGGTCAATGTTTGCCATCC (SEQ ID NO: 25)
5	Y373L	GACCTCTTTGATTGGTGGAGCTACTGATAG (SEQ ID NO: 26)
		ACCAATCAAAGAGGTCAATGTTTGCCATCC (SEQ ID NO: 27)
6	Y373C	GACCTCTTGTATTGGTGGAGCTACTGATAG (SEQ ID NO: 28)
		ACCAATACAAGAGGTCAATGTTTGCCATCC (SEQ ID NO: 29)
7	A175C	GGTGTGTACTGTGGAGATCCTCAACAGCTC (SEQ ID NO: 30)
		AGGATCTCCACAGTACACACCAGAAACAAA (SEQ ID NO: 31)
8	A175L	GGTGTGTACTTGGGAGATCCTCAACAGCTC (SEQ ID NO: 32)
		AGGATCTCCCAAGTACACACCAGAAACAAA (SEQ ID NO: 33)
9	V318M	TATCCAACAATGGCTTCAGTTGTGTTGGCA (SEQ ID NO: 34)
		AACTGAAGCCATTGTTGGATAAGTGAATGC (SEQ ID NO: 35)
10	G351A	CGCTGTCTCGCTACGATTGGACATCGAGT (SEQ ID NO: 36)
		CCAAATCGTAGCGAGACAGCGAATTCCCTG (SEQ ID NO: 37)
11	S63T	GCCCGAACACTTTTTCGCCGACGCCGAATTG (SEQ ID NO: 38)
		GGCGAAAAAGTGTTTCGGGCCCTCCTCCCAG (SEQ ID NO: 39)

12	R92A	CAAATTGCCTGCTTTTGTGTATTGGGAAAATAAG (SEQ ID NO: 40)
		ATACACAAAAGCAGGCAATTGCGATCGGC (SEQ ID NO: 41)
13	V173S	GTTTCTGGGAGTTATGCCGGCGATCCGCAAC (SEQ ID NO: 42)
		CCGGCATAACTCCCAGAAACAAAAGGTTCC (SEQ ID NO: 43)
14	V173C	GTTTCTGGGTGTTATGCCGGCGATCCGCAAC (SEQ ID NO: 44)
		CCGGCATAACACCCAGAAACAAAAGGTTCCAC (SEQ ID NO: 45)
15	V173T	GTTTCTGGGACTTATGCCGGCGATCCGCAAC (SEQ ID NO: 46)
		CCGGCATAAGTCCCAGAAACAAAAGGTTCCAC (SEQ ID NO: 47)
16	L229F	ACTAAGCCGGGGGAGTTCGGTTCGTTCAAGCAG (SEQ ID NO: 48)
		CTGCTTGAACGAACCGAACTCCCCCGGCTTAGT (SEQ ID NO: 49)
17	L340I	TTTTGGAAATATAATTCCGAGGGGGCAGGG (SEQ ID NO: 50)
		CTCGGAATTATATTCCAAAACCCACTAATTAC (SEQ ID NO: 51)
18	I353T	TCGGGACGACTTGGACATCGAGTTTATTTC (SEQ ID NO: 52)
		GATGTCCAAGTCGTCCCGAGACAGCGAATTC (SEQ ID NO: 53)
19	I353L	CTCGGGACGCTTTGGACATCGAGTTTATTT (SEQ ID NO: 54)
		ATGTCCAAAGCGTCCCGAGACAGCGAATTC (SEQ ID NO: 55)
20	I353V	ACTAAGCCGGGGGAGTTCGGTTCGTTCAAGCAG (SEQ ID NO: 56)
		CTGCTTGAACGAACCGAACTCCCCCGGCTTAGT (SEQ ID NO: 57)
21	I353C	TCGGGACG TGT TGGACATCGAGTTTATTTC (SEQ ID NO: 58)
		GATGTCCA ACA CGTCCCGAGACAGCGAATTC (SEQ ID NO: 59)
22	V318T	TATCCTACGACTGCCTCCGTTGTCTTAGCA (SEQ ID NO: 60)
		AACGGAGGCAGTCGTAGGATAAGTAAAAGC (SEQ ID NO: 61)
23	E228A	AAAATAAGCCGGGGGCGTTGGGTTTCGTTCAAG (SEQ ID NO: 62)
		CTTGAACGAACCCAACGCCCCCGGCTTAGTTTT (SEQ ID NO: 63)
24	P91L	AAACTTCTTAGATTCGTGTATTGGGAAAAC (SEQ ID NO: 64)
		ACGAATCTAAGAAGTTTTCTATCTGCGAAT (SEQ ID NO: 65)
25	P316A+V318L	GCTTTTACTTATGCTACGCTTGCTCCGTT (SEQ ID NO: 66)
		GACAACGGAGGCAAGCGTAGCATAAGTAAA (SEQ ID NO: 67)
26	P316L+V318L	GCTTTTACTTATCTIACGCTTGCTCCGTT (SEQ ID NO: 68)

		GACAACGGAGGCAAGCGTAAGATAAGTAAA (SEQ ID NO: 69)
27	F337V	TTAGTGGGTGTTGGAAATTTAATTCCGAGGGG (SEQ ID NO: 70)
		TAAATTCCAACACCCACTAATTTACCCTTGAC (SEQ ID NO: 71)
28	T352V	TGTCTCGGGGTGATTGGACATCGAGTTTATT (SEQ ID NO: 72)
		GTCCAAATCACCCCGAGACAGCGAATTCCTG (SEQ ID NO: 73)
29	V173L	GTTTCTGGGCTTTATGCCGCGATCCGCAAC (SEQ ID NO: 74)
		CGGCATAAAGCCCAGAAACAAAAGGTTCCAC (SEQ ID NO: 75)
30	A175I	GTTTCTGGGGTTTATATTGGCGATCCGCAA (SEQ ID NO: 76)
		TTGTTGCGGATCGCCAATATAAACCCAGAG (SEQ ID NO: 77)
31	L340V	TTTTGGAAATGTTATTCCAAGAGGTCAAGGAATC (SEQ ID NO: 78)
		CTTGGAATAACATTTCCAAAACCCACGAGTTT (SEQ ID NO: 79)
32	L340T	TTTTGGAAATACTATTCCAAGAGGTCAAGG (SEQ ID NO: 80)
		CTTGGAATAGTATTTCCAAAACCCACGAGTTTTC (SEQ ID NO: 81)
33	F169A	CCAGAAACAGCAGGTTCCACCAACCGCTGCATC (SEQ ID NO: 82)
		GTGGAACCTGCTGTTTCTGGGGTTTATGCCG (SEQ ID NO: 83)

【Table 3】

Primer for CyPPO4 mutation

No.	CyPPO4 mutation	Primer (Forward: top; Reverse: bottom) (5'→3')
1	Y375M	CTTACATCTATGATTGGTGGAGCTACCGAT (SEQ ID NO: 84)
		CCACCAATCATAGATGTAAGAACCTGCCAT (SEQ ID NO: 85)
2	Y375V	CTTACATCTGTTATTGGTGGAGCTACCGAT (SEQ ID NO: 86)
		CCACCAATAACAGATGTAAGAACCTGCCAT (SEQ ID NO: 87)
3	Y375I	CTTACATCTATCATTGGTGGAGCTACCGAT (SEQ ID NO: 88)
		CCACCAATGATAGATGTAAGAACCTGCCAT (SEQ ID NO: 89)
4	Y375T	CTTACATCTACCATTTGGTGGAGCTACCGAT (SEQ ID NO: 90)
		CCACCAATGGTAGATGTAAGAACCTGCCAT (SEQ ID NO: 91)
5	Y375C	CTTACATCTTGTATTGGTGGAGCTACCGAT (SEQ ID NO: 92)

		CCACCAATACAAGATGTAAGAACCTGCCAT (SEQ ID NO: 93)
6	A176C	GGTGTGTATTGTGGAGATCCACAACAGCTT (SEQ ID NO: 94)
		TGGATCTCCACAATACACACCAGAAACAAA (SEQ ID NO: 95)
7	A176L	GGTGTGTATTTGGGAGATCCACAACAGCTT (SEQ ID NO: 96)
		TGGATCTCCCAAATACACACCAGAAACAAA (SEQ ID NO: 97)
8	P318L+V320L	ATTCCTTATTTGCCATTGGCTTGTGTTGTGCTC (SEQ ID NO: 98)
		AACACAAGCCAATGGCAAATAAGGAATTTCTGTA (SEQ ID NO: 99)
9	V320M	TATCCTCCAATGGCTTGTGTTGTGCTCGCA (SEQ ID NO: 100)
		AACACAAGCCATTGGAGGATAAGGAATTTCTGTA (SEQ ID NO: 101)
10	P318A+V320L	ATTCCTTATGCTCCATTGGCTTGTGTTGTGCTC (SEQ ID NO: 102)
		AACACAAGCCAATGGAGCATAAGGAATTTCTGTA (SEQ ID NO: 103)

【Table 4】

Primer for CyPPO8 mutation

No.	CyPPO8 mutation	Primer (Forward: top; Reverse: bottom)
1	F363M	TTGACAAATATGATTGGTGGAGCTACCGAT (SEQ ID NO: 104)
		CACCAATCATATTTGTCAAAAGGTGCCATC (SEQ ID NO: 105)
2	F363V	CTT TTG ACA AAT GTT ATT GGT GGA GCT ACC (SEQ ID NO: 106)
		AGC TCC ACC AAT AAC ATT TGT CAA AAG GTG (SEQ ID NO: 107)
3	F363L	CTT TTG ACA AAT CTT ATT GGT GGA GCT ACC (SEQ ID NO: 108)
		AGC TCC ACC AAT AAG ATT TGT CAA AAG GTG (SEQ ID NO: 109)
4	F363C	CTT TTG ACA AAT TGT ATT GGT GGA GCT ACC (SEQ ID NO: 110)
		AGC TCC ACC AAT ACA ATT TGT CAA AAG GTG (SEQ ID NO: 111)
5	A162C	TCTGGTGTGTAT TGT GGAGATGTTGATCAA (SEQ ID NO: 112)
		ATCAACATCTCC ACA ATACACACCAGAAAC (SEQ ID NO: 113)
6	A162L	TCTGGTGTGTAT CTT GGAGATGTTGATCAA (SEQ ID NO: 114)
		ATCAACATCTCC AAG ATACACACCAGAAAC (SEQ ID NO: 115)
7	P306L+V308L	ATCTCATAT CTT CCA CTT GCTTGC GTTGTG (SEQ ID NO: 116)

		AACGCAAGC AAG TGG AAG ATATGAGATTTC (SEQ ID NO: 117)
8	V308M	TCATATCCTCCA ATG GCTTGC GTTGTGCTC (SEQ ID NO: 118)
		CACAACGCAAGC CAT TGGAGGATATGAGAT (SEQ ID NO: 119)
9	P306A+V308L	ATTTCCTATGCCCCCTAGCTTGTGTGGTCTTAGCC (SEQ ID NO: 120)
		CACACAAGCTAGGGGGGCATAGGAAATTCGTTTAAGG (SEQ ID NO: 121)
10	V160S	GTGTCTGGGTCTTATGCAGGGGATGTGGATC (SEQ ID NO: 122)
		CCTGCATAAGACCCAGACACAAACGGACTCAC (SEQ ID NO: 123)
11	I343T	TTGGTACAACCTGGAGTTCAACACTCTTCC (SEQ ID NO: 124)
		GAACTCCAAGTTGTACCAAGAGTGCGGATTC (SEQ ID NO: 125)
12	F363I	CTT TTG ACA AAT ATT ATT GGT GGA GCT ACC (SEQ ID NO: 126)
		AGC TCC ACC AAT AAT ATT TGT CAA AAG GTG (SEQ ID NO: 127)
13	F363T	CTT TTG ACA AAT ACT ATT GGT GGA GCT ACC (SEQ ID NO: 128)
		AGC TCC ACC AAT AGT ATT TGT CAA AAG GTG (SEQ ID NO: 129)
14	R85A	GGACTTCCCGCTTATGTTTATTGGGAGGGG (SEQ ID NO: 130)
		CAATAAACATAAGCGGGAAGTCCGCGATCAGC (SEQ ID NO: 131)
15	I343V	CTTGGTACAGTTTGGAGTTCAACACTCTTTC (SEQ ID NO: 132)
		AACTCCAAACTGTACCAAGAGTGCGGATTC (SEQ ID NO: 133)
16	P84L	AGGTTTACTTAGGTATGTTTACTGGGAGGG (SEQ ID NO: 134)
		CATACCTAAGTAAACCTCTATCTGCAAAGA (SEQ ID NO: 135)
17	R85C	GACTTCCCTGTTATGTTTATTGGGAGGGGAAAC (SEQ ID NO: 136)
		TAAACATAACAGGGAAGTCCGCGATCAGCAAAG (SEQ ID NO: 137)
18	R85H	ACTTCCCCATTATGTTTATTGGGAGGGGAAAC (SEQ ID NO: 138)
		CAATAAACATAATGGGGAAGTCCGCGATCAGC (SEQ ID NO: 139)
19	R85L	ACTTCCCCTTTATGTTTATTGGGAGGGGAAAC (SEQ ID NO: 140)
		CAATAAACATAAAGGGGAAGTCCGCGATCAGC (SEQ ID NO: 141)
20	R85T	GACTTCCCCTTATGTTTATTGGGAGGGGAAAC (SEQ ID NO: 142)
		ATAAACATAAGTGGGAAGTCCGCGATCAGCAAAG (SEQ ID NO: 143)
21	R85V	GACTTCCCGTTTATGTTTATTGGGAGGGGAAAC (SEQ ID NO: 144)

		ATAAACATAAACGGGAAGTCCGCGATCAGCAAAG (SEQ ID NO: 145)
22	F156A	GTGAGTCCGGCTGTGTCTGGGGTTTATGCA (SEQ ID NO: 146)
		CCCAGACACAGCCGGACTCACTAACCGTTG (SEQ ID NO: 147)
23	V160C	CCGTTTGTGTCTGGGTGCTATGCAGGGGATGTG (SEQ ID NO: 148)
		CACATCCCCTGCATAGCACCCAGACACAAACGG (SEQ ID NO: 149)
24	Q179G	TCCGCCAGTCCGGTAACTCGTCCAAATGCAG (SEQ ID NO: 150)
		CGAGTTACCGGACTGGCGGATGTGGGCGGTG (SEQ ID NO: 151)
25	F327V	GTTTTCCCCTCAAAGGAGTGGGTAACTTAACCCTC (SEQ ID NO: 152)
		GAGGGTTAAGATTACCCACTCCTTTGAGGGGAAAAC (SEQ ID NO: 153)
26	L330T	TTTGGAATACTAACCCTCGCAGTCAAGGA (SEQ ID NO: 154)
		GCGAGGGTTAGTATTACCAAATCCTTTGAG (SEQ ID NO: 155)
27	F363M+N362S	CTTTTGACATCAATGATTGGTGGAGCTACC (SEQ ID NO: 156)
		CCAATCATTGATGTCAAAAGGTGCCATCCT (SEQ ID NO: 157)

Single colony of *E. coli* transformed with each CyPPO gene was cultured in 3 ml of LB broth containing chloramphenicol for overnight (220 rpm, 37 °C), and then the each culture was re-cultured with fresh media until absorbance (OD₆₀₀) became 0.5 to 1. It was diluted with LB broth to absorbance (OD₆₀₀) of 0.5. Again, the diluted solution was serially diluted 5 times by a factor of one tenth. Next, 10 µl of each diluted solution was dropped on the LB agar media containing chloramphenicol (34 µg/ml) and 0~400 µM concentration of tiafenacil. The LB agar media were cultured at 37 °C under light condition, and level of growth inhibition was evaluated after 16 to 20 hours.

Herbicides used in the test were listed in Table 5:

10 【Table 5】

Family	Herbicide
Pyrimidinedione-based herbicide	Tiafenacil
	Saflufenacil
Diphenyl ether-based herbicide	Fomesafen
	Acifluorfen

N-phenylphthalimides-based herbicide	Flumioxazin
Triazolinones-based herbicide	Sulfentrazone
Oxazolidinediones-based herbicide	Pentoxazone
Phenylpyrazoles-based herbicide	Pyraflufen-ethyl
Others	Pyraclonil

The results were shown in Tables 6 to 8 and FIGs. 5 to 25.

【Table 6】

Herbicide tolerance level conferred by CyPPO2 mutation

CyPPO2	Tiafenacil	Saflufenacil	Flumioxazin
CyPPO2 (wild type)	-	-	-
Y373M+G351A	+++++	+++++	+++++
Y373C	++++	+++++	+++++
Y373I	++++	+++++	+++++
Y373L	++++	+++++	+++++
Y373M	++++	+++++	+++++
Y373T	++++	+++++	+++++
Y373V	+++	+++++	+++++
A175C	+++	++++	+++++
A175L	++	+++++	++++
V318M	++	++++	+++++
P316A+V318L	+	NT	NT
P316L+V318L	+	NT	NT
F337V+Y373M	++	NT	NT
T352V+Y373M	++++	NT	NT
G351A+T352V+Y373M	+++	NT	NT
P91L+Y373M	++	NT	NT

5 (NT: Not Tested)

【Table 7】

Herbicide tolerance level conferred by CyPPO4 mutation

CyPPO4	Tiafenacil	Saflufenacil	Flumioxazin
CyPPO4	-	-	-
Y375M	+++++	+++++	+++++
Y375V	+++++	+++++	+++++
Y375I	+++++	+++++	+++++
Y375T	+++++	+++	+++++
Y375C	+++++	+++	NT
A176C	+++++	+++++	+++++
A176L	+++++	+++++	+++++
P318L+V320L	+++++	++++	NT
V320M	+++++	++++	NT
P318A+V320L	+++++	++++	NT

(NT: Not Tested)

【Table 8】

5

Herbicide tolerance level conferred by CyPPO8 mutation

CyPPO8	Tiafenacil	Saflufenacil	Fomesafen	Acifluorfen	Flumioxazin	Sulfentrazone	Pentoxazone	Pyraflufen-ethyl	Pyraclonil
CyPPO8	-	-	-	-	-	-	-	-	-
F363C	++++	++++	++++	+++++	+++	++++	+++++	++++ +	+++++
F363L	+++	+++	+	+++	++	++	+++++	++++	++++
F363M	+++	+++	+	+++	++	++	+++++	++++	++++
F363V	+++	+++	-	++	+++	++	+++++	++++	+++++
A162L	++	++	+++	++++	+++	++++	++++	++++	+++
V308M	++	++	+++	+++++	+++	++++	++++	++++	++++
P306A+ V308L	+	+	+	++++	NT	++	+++	+++	+

P306L+ V308L	+	+	++	+++++	NT	++++	+++	++++	+
A162C	NT	NT	NT	++	NT	+	+++	++++	+
P84L+F 363M	+++	NT	NT	NT	NT	NT	NT	NT	NT
N362S+ F363M	+++	NT	NT	NT	NT	NT	NT	NT	NT

(NT: Not Tested)

The Tables 6 to 8 show the results of evaluating the herbicide tolerance of wild type and PPO variants. The level of herbicide tolerance of the wild type was represented by "-", and the level of herbicide tolerance was graduated by representing the equal level of tolerance by "-", and if higher, adding "+" to the max "+++++".

FIGs 5 to 25 show the results of *E. coli* transformed with the CyPPO genes (wild type and variants), and the concentration of herbicide treatment is described on the top. The most left column is the result of *E. coli* culture solution OD₆₀₀=0.5, and the next five columns were diluted 5 times by a factor of one tenth.

As shown in Table 6 and FIGs. 5 to 13, CyPPO2 wild type transformant (hereinafter, represented by 'Cy2 WT'; control) showed rapid growth inhibition from 25μM or 50μM of pyrimidinedione-based herbicide tiafenacil treatment, but growth inhibition of transformant wherein mutant genes of Y373C, Y373I, Y373L, Y373M, Y373T, Y373V, A175C, A175L, V318M, G351A+Y373M, P316A+V318L, P316L+V318L, Y373M+F337V, Y373M+T352V, Y373M+G351A+T352V, and Y373M+P91L were introduced respectively was not observed even at the maximum concentration (200 μM) (FIG. 5). When another pyrimidinedione-based herbicide saflufenacil was treated, Cy2 WT also showed growth inhibition at 100 μM or the higher concentration, but the transformant wherein mutant genes of Y373C, Y373I, Y373L, Y373M, Y373T, Y373V, A175C, A175L, V318M, and G351A+Y373M were introduced respectively was not observed even at the maximum concentration (200 μM) (FIG. 6). When N-phenylphthalimides-based herbicide flumioxazin was treated, the growth inhibition of Cy2 WT was started from 25μM of concentration of herbicide

treatment, but the growth inhibition of the transformant wherein mutant genes of Y373C, Y373I, Y373L, Y373M, Y373T, Y373V, A175C, A175L, V318M, and G351A+Y373M were introduced respectively was not observed at the maximum concentration (200 μ M) (FIG. 7).

5 As shown in Table 7 and FIGs. 14 to 16, when tiafenacil was treated, CyPPO4 wild type gene was introduced (hereinafter, represented by 'Cy4 WT'; control) showed growth inhibition from 100 μ M of the herbicide treated, but the growth inhibition of transformant wherein mutant genes of A176L, A176C, P318L+V320L, P318A+V320L, V320M, Y375I, Y375T, Y375V, Y375M, and Y375C were introduced respectively was not observed at the maximum concentration (200 μ M) (FIG. 14). When saflufenacil was treated, Cy4 WT showed growth inhibition from 100 μ M of the herbicide treatment, but the growth inhibition of transformant wherein mutant genes of Y375C, Y375I, Y375M, Y375T, Y373V, A176C, A176L, P318L+V320L, P318A+V320L, and V320M were introduced respectively was not observed at the maximum concentration (200 μ M) (FIG. 15). When flumioxazin was treated, the growth inhibition of Cy4 WT was observed from 200 μ M of concentration of herbicide treatment, but the growth inhibition of the transformant wherein mutant genes of Y375I, Y375M, Y375T, Y373V, A176C, and A176L were introduced respectively was not observed (FIG. 16).

As shown in Table 8 and FIGs. 17 to 25, when tiafenacil or saflufenacil was treated, CyPPO8 wild type gene was introduced (hereinafter, represented by 'Cy8 WT'; control) showed no growth from 5 μ M of herbicide treatment concentration, the growth of the transformant wherein mutant genes of F363C, F363L, F363M, F363V, A162L, V308M, P306A+V308L, P306L+V308L, F363M+P84L, and F363M+N362S were introduced respectively was observed even at the minimum concentration of 25 μ M or higher (FIGs. 17 and 18). When diphenyl-ether-based herbicide fomesafen was treated, Cy8 WT showed no growth from 25 μ M of herbicide treatment concentration, but the growth of the transformant wherein mutant genes of F363C, F363L, F363M, A162L, V308M, P306A+V308L, and P306L+V308L were introduced respectively was observed at the concentration of 25 μ M or higher (FIG. 19). When another diphenyl-ether-based herbicide acifluorfen was treated, Cy8 WT showed no growth from 50 μ M

of herbicide treatment concentration, but the growth of the transformant wherein mutant genes of F363C, F363L, F363M, F363V, A162C, A162L, V308M, P306A+V308L, and P306L+V308L were introduced respectively was observed at the concentration of 50 μ M or higher (FIG. 20). When flumioxazin was treated, Cy8 WT showed no growth from 5 μ M of herbicide treatment concentration, but the growth of the transformant wherein mutant genes of F363C, F363L, F363M, F363V, A162L, and V308M were introduced respectively was observed at the concentration of 25 μ M or higher (FIG. 21). When triazolinones-based herbicide sulfentrazone was treated, Cy8 WT showed growth only by 25 μ M of herbicide treatment concentration, but the growth of the transformant wherein mutant genes of F363C, F363L, F363M, F363V, A162C, A162L, V308M, P306A+V308L, and P306L+V308L were introduced respectively was observed at the concentration of 50 μ M or higher (FIG. 22). When pentoxazone or pyraflufen-ethyl was treated, Cy8 WT showed no growth from 5 μ M of herbicide treatment concentration, but the growth of the transformant wherein mutant genes of F363C, F363L, F363M, F363V, A162C, A162L, V308M, P306A+V308L, and P306L+V308L were introduced respectively was observed at the maximum concentration (200 μ M) (FIGs. 23 and 24). When pyraclonil was treated, Cy8 WT showed no growth from 25 μ M of herbicide treatment concentration, but the growth of the transformant wherein mutant genes of F363C, F363L, F363M, F363V, A162C, A162L, V308M, P306A+V308L, P306L+V308L were introduced respectively was observed at the maximum concentration (200 μ M) (FIG. 25).

Example 4: Measurement of enzyme activity and IC₅₀ value by herbicides of PPO wild type and variants

The enzyme activity of variants wherein amino acids of certain position of PPO protein were mutated to increase herbicide tolerance was examined and inhibition assay for the PPO-inhibiting herbicides was conducted. It was confirmed that the PPO protein had low water-solubility, but when the PPO protein was fused with MBP (maltose binding protein) (MBP-PPO), it was water-soluble and stably expressed. Therefore, the

wild type and variant proteins which were expressed in the form of fusion protein with MBP were used in the present experiments (refer to FIG. 26).

In order to clone wild type genes and variant genes of CyPPO2, CyPP04, and CyPPO8 (refer to Example 1 and Example 2), those genes were introduced into pMAL-c2X vector (refer to FIG. 27) respectively, and then expressed in BL21-CondonPlus (DE3) *E. coli* (CodonPlus).

The transformed *E. coli* were cultured under the following conditions to express introduced PPO genes:

Induction: OD₆₀₀=0.2, addition of IPTG to 0.3 mM final concentration;

10 Expression temperature: 23 °C, 200rpm shaking culture;

Expression time: 16 hrs;

Culture scale: 200 ml/1,000 ml flask.

Cell lysis and protein extraction were performed by the following process to the cultured cells:

15 Extraction buffer: Column buffer (50 mM Tris-Cl, pH 8.0, 200 mM NaCl) 5 ml buffer/g cell;

Sonication: SONICS&MATERIALS VCX130 (130 watts);

15 sec ON, 10 sec OFF for 5 min on ice;

20 Centrifugation under the condition of 4°C for 20 minutes (20,000 xg); and the supernatant obtained by the centrifugation was diluted at the ratio of 1:6 using column buffer.

The following process for purification of PPO protein was performed in a 4°C cold room. Amylose resin (New England Biolabs) was packed to 1.5x15 cm column (Bio-Rad Econo Columns 1.5 x 10 cm, glass chromatography column, max. vol), and the obtained protein extracts were loaded to the column at the a flow rate of 0.2 ml/min. The column was washed with 3 column volumes of buffer and the amount of protein in the washing solution was checked. When the protein was no longer detected, the washing was terminated. Then, the MBP-PPO protein was eluted with approximately 2 column volumes of buffer containing 20 mM maltose. The protein concentration of each eluent was determined and the elution was stopped when the protein was no longer

detected. Ten microliter of each fraction was investigated for protein quantification and SDS-PAGE analysis. The highly pure fractions of PPO protein variants (for example, CyPPO2-Y373M, CyPPO4-Y375M, CyPPO8-F363M) were taken for the enzyme assay.

The SDS-PAGE analysis of each PPO was shown in FIGs. 28 to 30. Referring to the result shown in FIG. 28, CyPPO2 wild type protein (elution 7, lane 2) and CyPPO2-Y373M (elution 5, lane 7) were taken and used for analysis of enzyme activity.

Referring to the result shown in FIG. 29, as to CyPPO4 wild type protein (elution 3, lane 2) and CyPPO4-Y375M (elution 3, lane 6) were taken and used for analysis of enzyme activity.

Referring to the result shown in FIG. 30, CyPPO8 wild type protein (elution 9, lane 3) and CyPPO8-F363M (elution 4, lane 6) were taken and used for analysis of enzyme activity.

The enzyme activity of the purified wild type protein and variant proteins of CyPPO2, CyPPO4 and CyPPO8 was measured by the following process.

Overall process was performed in dark under nitrogen stream. Since protoporphyrinogen IX, a substrate of PPO protein was not commercially available, it was chemically synthesized in the laboratory. Six micrograms of protoporphyrin IX was dissolved in 20 ml of 20% (v/v) EtOH, and stirred under dark condition for 30 minutes. The obtained protoporphyrin IX solution was put into a 15 ml screw tube in an amount of 800 μ l, and flushed with nitrogen gas for 5 minutes. To this, 1 g of sodium amalgam was added and vigorously shaken for 2 minutes. The lid was opened to exhaust hydrogen gas in the tube. Thereafter, the lid was closed and incubated for 3 minutes. The protoporphyrinogen IX solution was filtered using syringe and cellulose membrane filter. To 600 μ l of the obtained protoporphyrinogen IX solution, approximately 300 μ l of 2M MOPS [3-(N-morpholino)propanesulfonic acid] was added to adjust pH to 8.0. To determine the enzyme activity of PPO protein, a reaction mixture was prepared with the following composition (based on 10 ml): 50 mM Tris-Cl (pH 8.0); 50 mM NaCl; 0.04% (v/v) Tween 20; 40 mM glucose (0.072 g); 5 units glucose oxidase (16.6 mg); and 10 units catalase (1 μ l).

Two hundred microliters of a reaction mixture containing a purified PPO protein was placed in 96 well plates and preincubated for 30 min at room temperature to reduce the oxygen concentration by the coupled reaction of glucose oxidase-catalase. The mineral oil was layered and then the reaction was initiated by adding the substrate, protoporphyrinogen IX solution to a final concentration of 50 μ M. The reaction proceeded at room temperature for 30 min and the fluorescence of protoporphyrin IX was measured using Microplate reader (Sense, Hidex) (excitation: 405 nm; emission: 633 nm). To calculate the PPO enzyme activity, the protoporphyrinogen IX solution was kept open in the air to oxidize the solution (overnight). To this, 2.7 N HCl was added, and the absorbance at 408 nm was measured. A standard curve was generated using standard protoporphyrin IX, and PPO activity was measured by calibration of protoporphyrin IX using the standard curve of protoporphyrin IX.

The enzyme activity of the obtained PPO wild type and variants was shown in Tables 10 and 11.

Michaelis-Menten constant (K_m) and the maximal velocity (V_{max}) values of each enzyme were calculated in order to evaluate the kinetic study of CyPPO2 and CyPPO8. The initial reaction velocity was measured where the reaction velocity was proportional to concentration by varying the substrate concentration. The amount of produced protoporphyrin IX which is an enzyme reaction product was measured by time course at room temperature for 20 minutes. K_m and V_{max} values were calculated with the enzyme kinetics analysis program by Michaelis-Menten equation. The wild type AtPPO1 was used as a control. The result was shown in Table 9:

【Table 9】

Determination of K_m and V_{max} of CyPPO2 and CyPPO8

	CyPPO2	CyPPO8	AtPPO1
$K_m(\mu M)$	9.1 ± 0.5	7.7 ± 0.2	9.6 ± 1.8
V_{max} (μM mg protein-1 min-1)	285 ± 15	305 ± 10	135 ± 19

From the results, K_m values of CyPPO2 and CyPPO8 were lower than that of

AtPPO1, confirming that the affinity between enzyme and substrate was better, while Vmax values of CyPPO2 and CyPPO8 were more than two times higher than that of AtPPO1. In conclusion, PPO proteins of CyPPO2 and CyPPO8 show better ability as PPO enzyme than the plant-derived AtPPO1.

5 In addition, the concentration of the PPO-inhibiting herbicides that inhibits the PPO enzyme activity of each PPO wild type and variants by 50% (IC₅₀) was measured for each herbicide. The final concentrations of each herbicide were as follows:

- Tiafenacil, saflufenacil, fomesafen, butafenacil, flumioxazin and sulfentrazone:

0, 10, 50, 100, 250, 500, 1,000, 2,500, 5,000 nM

10 The IC₅₀ value was calculated as the concentration of the herbicide inhibiting the PPO enzyme activity to 50% before adding the herbicide at the above concentration.

The IC₅₀ values of different herbicides were shown in the following Tables 10 and 11.

【Table 10】

CyPP O2	Mutation site	Acti vity (%)	IC ₅₀ (nM)					
			Tiafenacil	Saflufenacil	Fomesafen	Butafenacil	Flumioxazin	Sulfentrazone
	WT	100	25	50	182	13	122	NT
1	Y373M	90	250	5,000	395	63	136	NT
2	Y373I	75	639	5,000	70	1516	464	NT
3	Y373L	44	300	NT	NT	NT	NT	NT
4	Y373V	35	525	NT	NT	NT	NT	NT
5	Y373C	39	225	NT	NT	NT	NT	NT
6	Y373T	10	2500	NT	NT	NT	NT	NT
7	A175L	41	230	NT	NT	NT	NT	NT
8	S63T+ Y373M	90	246	NT	NT	NT	NT	NT
9	R92A+ Y373M	50	219	NT	NT	NT	310	NT

10	V173S+ Y373M	80	430	NT	NT	NT	NT	NT
11	V173S+ Y373I	25	610	NT	NT	NT	NT	NT
12	V173S+ Y373L	40	530	NT	NT	NT	NT	NT
13	V173S+ Y373V	10	1,000	NT	NT	NT	NT	NT
14	V173C+ Y373M	53	680	NT	NT	NT	NT	NT
15	V173C+ Y373I	28	915	NT	NT	NT	NT	NT
16	V173T+ Y373M	15	543	NT	NT	NT	NT	NT
17	V173T+ Y373I	25	1,500	NT	NT	NT	NT	NT
18	V173L+ Y373M	83	290	NT	NT	NT	NT	NT
19	A175L+ Y373M	62	4,500	5,000	1,720	4,000	5,000	5,000
20	A175L+ Y373I	15	2,000	5,000	NT	NT	NT	NT
21	A175C+ Y373M	83	3,700	5,000	770	2,500	372	5,000
22	A175C+ Y373I	31	2,000	NT	NT	NT	NT	NT
23	A175I+ Y373M	52	2,500	NT	NT	NT	NT	NT
24	E228A+	85	214	NT	NT	NT	NT	NT

	Y373M							
25	L229F+ Y373T	10	1,800	NT	NT	NT	NT	NT
26	V318M+ Y373M	73	456	5,000	523	708	210	3776
27	V318M+ Y373I	50	2,765	5,000	145	1,232	1,021	5,000
28	V318M+ Y373V	42	2,140	5,000	NT	NT	NT	NT
29	V318T+ Y373I	25	1,500	NT	NT	NT	NT	NT
30	L340I+ Y373M	80	259	NT	NT	NT	NT	NT
31	L340I+ Y373I	85	584	NT	NT	NT	NT	NT
32	L340V+ Y373M	80	257	NT	NT	NT	NT	NT
33	G351A+ Y373M	23	706	5,000	300	36	NT	NT
34	I353T+ Y373M	32	1,152	NT	NT	NT	NT	NT
35	I353T+ Y373I	20	1,300	NT	NT	NT	NT	NT
36	I353T+ Y373L	10	3,000	NT	NT	NT	NT	NT
37	I353L+ Y373M	80	271	NT	NT	NT	NT	NT
38	I353V+ Y373M	63	445	NT	NT	NT	NT	NT

39	I353C+ Y373M	12	550	NT	NT	NT	NT	NT
40	S63T+ V173S+ Y373M	60	430	NT	NT	NT	NT	NT
41	S63T+ V173S+ Y373I	60	427	NT	NT	NT	NT	NT
42	S63T+ I353T+ Y373M	15	278	NT	NT	NT	NT	NT
43	S63T+ I353T+ Y373I	5	1200	NT	NT	NT	NT	NT
44	V173S+ V318M+ Y373M	5	3,000	NT	NT	NT	NT	NT
45	V173T+ L340I+ Y373M	50	518	NT	NT	NT	NT	NT
46	V173S +A175C +Y373M	38	5,000	5,000	758	2,591	5,000	5,000
47	A175C+ V318M+ Y373M	47	3,793	5,000	2,033	1,475	419	5,000
48	A175L+ V318M+ Y373M	40	3,100	NT	NT	NT	NT	NT
49	A175C+	5	1,900	NT	NT	NT	NT	NT

	I353L+ Y373M							
50	A175C+ I353V+ Y373M	69	2,310	NT	NT	NT	NT	NT
51	R92A	66	125	NT	NT	NT	NT	NT
52	F169A	72	57	NT	NT	NT	NT	NT
53	V173C	68	79	NT	NT	NT	NT	NT
54	A175C	86	100	NT	NT	NT	NT	NT
55	A175L	68	325	NT	NT	NT	NT	NT
56	V318M	76	140	NT	NT	NT	NT	NT
57	F337V	20	148	NT	NT	NT	NT	NT
58	L340T	18	56	NT	NT	NT	NT	NT
59	I353T	40	162	NT	NT	NT	NT	NT
CyPP O4								
	WT	100	22	33	28	10	NT	NT
1	Y375M	95	124	5,000	133	39	NT	NT

(NT: not tested)

【Table 11】

CyP PO8 No.	Mutation site	Acti vity (%)	IC ₅₀ (nM)					
			Tiafenacil	Saflufena cil	Fomesafe n	Butafenac il	Flumioxa zin	Sulfentra zone
	WT	100	4	11	13	2.8	NT	NT
1	F363M	100	183	5,000	163	30	266	NT
2	F363C	17	36	500	38	53	NT	NT
3	F363V	15	2,000	5,000	1,500	250	2,500	NT
4	F363L	54	322	NT	NT	NT	NT	NT

5	F363I	62	698	5,000	133	NT	1100	NT
6	F363T	5	3,000	5,000	1,500	NT	2,500	NT
7	R85A + F363M	62	700	NT	NT	NT	NT	NT
8	R85A + F363I	40	2,350	NT	NT	NT	NT	NT
9	A162L + F363M	17	5,000	NT	NT	NT	5,000	NT
10	A162C + F363M	87	2,500	5,000	NT	NT	1,166	NT
11	V160S + F363M	58	500	NT	NT	NT	NT	NT
12	V160S + F363I	26	2,280	NT	NT	NT	NT	NT
13	V308M + F363M	81	1,017	5,000	NT	NT	210	NT
14	V308M + F363I	29	1,810	NT	NT	NT	NT	NT
15	I343T + F363M	10	2,100	NT	NT	NT	NT	NT
16	I343V + F363M	44	288	NT	NT	NT	NT	NT
17	R85A + V308M + F363M	39	2,347	5,000	NT	NT	NT	NT
18	V160S + V308M + F363I	5	4,000	NT	NT	NT	NT	NT
19	A162C + F363I	29	2,450	NT	NT	NT	NT	NT

20	A162C + F363L	6	5,000	NT	NT	NT	NT	NT
21	R85A + A162L + F363M	11	5,000	NT	NT	NT	NT	NT
22	R85A + A162L + F363I	15	1,061	NT	NT	NT	NT	NT
23	R85A + A162C + F363M	19	5,000	5,000	5,000	4,310	2,700	5,000
24	R85A + A162C + F363I	16	5,000	NT	NT	NT	NT	NT
25	A162C + V308M + F363M	45	3,580	5,000	420	2500	697	5,000
26	A162C + V308L + F363M	53	2,041	NT	NT	NT	NT	NT
27	R85C + F363M	28	2,500	NT	NT	NT	NT	NT
28	R85H + F363M	30	622	NT	NT	NT	NT	NT
29	R85L + F363M	19	892	NT	NT	NT	NT	NT
30	R85T + F363M	24	1,356	NT	NT	NT	NT	NT
31	R85V + F363M	18	875	NT	NT	NT	NT	NT

32	A162L + Q179G + F363M	39	5,000	5,000	630	4,000	5,000	5,000
33	R85A	98	80	NT	NT	NT	NT	NT
34	F156A	85	28	NT	NT	NT	NT	NT
35	V160C	80	44	NT	NT	NT	NT	NT
36	A162C	76	75	NT	NT	NT	NT	NT
37	A162L	82	219	NT	NT	NT	NT	NT
38	V308M	93	76	NT	NT	NT	NT	NT
39	F327V	22	81	NT	NT	NT	NT	NT
40	L330T	27	69	NT	NT	NT	NT	NT
41	I343T	18	365	NT	NT	NT	NT	NT

(NT: not tested)

As shown in the Tables 10 and 11, it was demonstrated that in case of variants of CyPPO proteins, the IC₅₀ values of each herbicide were significantly increased compared to the wild type. Such results show that herbicide tolerance was increased by amino acid mutation at certain positions of PPO protein. Although the present data showed that CyPPO protein variants have reduced enzyme activity compared to the wild type, it might be caused by the different conditions of the protein folding, and/or hydrophobicity of recombinants PPOs compared to the native PPOs. While the native PPOs are hydrophobic and localize to the membranes of chloroplasts in plants, the recombinant PPOs produced in *E. coli* are hydrophilic containing a MBP as a fusion partner. Thus, when PPO variants are properly assembled and expressed to chloroplasts in plants, the enzyme activity would not be affected drastically.

Example 5. Generation of *A. thaliana* transformants using CyPPO variants and PPO-inhibiting herbicide tolerance test

5-1. Construction of *A. thaliana* transformant vectors and generation of *A. thaliana* transformants

A. thaliana was transformed with a binary vector having ORF of a selectable

marker, Bar gene (glufosinate-tolerant gene) and ORF of each amino acid mutation gene of CyPPO2, CyPPO4 or CyPPO8. The transgenic plant was examined for cross-tolerance towards glufosinate and PPO-inhibiting herbicides. The bar gene was also used to examine whether the transgene was stably inherited during generations. NOS promoter and E9 terminator were used for bar gene expression.

In order to express CyPPO2 variants, CyPPO4 variants, and CyPPO8 variants, respectively in a plant, a CaMV35S promoter and NOS terminator were used. In addition, in order to transit protein to chloroplast, transit peptide (TP) of AtPPO1 gene (SEQ ID NO: 8) was inserted in front of 5' of the inserted gene using XbaI and XhoI restriction enzymes. Further, for identification of the expressed protein, hemagglutinin (HA) tag was fused to the 3'-terminal region using BamHI and SacI restriction enzymes. The transit peptide region inserted into the vector was represented by SEQ ID NO: 10, and the inserted HA tag sequence was represented by SEQ ID NO: 11. Encoding genes of CyPPO2 variants, CyPPO4 variants, or CyPPO8 variants were inserted between the transit peptide and HA tag using XhoI and BamHI restriction enzymes. NOS terminator was inserted after HA tag, thereby terminating transcription of PPO gene. A schematic diagram of the plant transformation binary vector is shown in FIG. 31.

Each constructed vector was transformed to *Agrobacterium tumefaciens* GV3101 competent cell by a freeze-thaw method. To prepare *Agrobacterium* GV3101 competent cell, *Agrobacterium* GV3101 strain was cultured in 5 ml LB media under the condition of 30 °C and 200 rpm for 12 hrs. The culture solution was poured to 200 ml LB media, and then cultured under the condition of 30 °C and 200 rpm for 3~4 hrs, and centrifuged at 4 °C for 20 minutes. The cell pellet was washed with sterile distilled water, and then resuspended in 20 ml LB media. Snap frozen 200ul aliquots with liquid nitrogen were stored in a deep freezer.

Each transformed *Agrobacterium* was cultured in an antibiotic media (LB agar containing spectinomycin) and screened. The screened colony was liquid cultured in LB broth. After *Agrobacterium* cell was harvested from the culture solution, it was resuspended in a 5% (w/v) sucrose, 0.05% (v/v) Silwet L-77 solution (Momentive performance materials company) at an absorbance (OD₆₀₀) of 0.8. By floral dipping

method, *A. thaliana* wild type (Col-0 ecotype) was transformed, and then the seed (T_0) was harvested 1~2 months later.

Bar gene in the binary vector was used for screening of individual transformants. The obtained T_0 seeds were sown in 1/2 MS media (2.25 g/L MS salt, 10 g/L sucrose, 7 g/L Agar) supplemented with 25 μ M glufosinate, and the surviving plants were selected after 7 days of sowing, and transplanted into soil and grown, to obtain T_1 plants.

In order to examine PPO-inhibiting herbicide tolerance of the transgenic plants, 4-week-old plants were evenly sprayed with 100ml of 1uM Tiafenacil solution (0.05% Silwet L-77) per 40 x 60 cm area (0.24 m²). While wild type *A. thaliana* (Col-0 ecotype) completely died within 7 days after treatment, each transformant showed no damage to PPO-inhibiting herbicide treatment.

The T_2 seeds were sown to 1/2 MS media (2.25 g/L MS salt, 10 g/L sucrose, 7 g/L Agar) supplemented with 25 μ M glufosinate, and after 1 week, surviving plants were transplanted into soil.

The *A. thaliana* wild type PPO1 (wild type AtPPO1) was used as a negative control having PPO-inhibiting herbicides sensitivity (GenBank accession no. AX084732 (the nucleotide sequence of the gene was represented by SEQ ID NO: 8, and the amino acid sequence was represented by SEQ ID NO: 7)). Mutant AtPPO1 with amino acid substitutions of Y426M (the 426th amino acid, tyrosine was substituted with methionine) and S305L (the 305th amino acid, serine was substituted with leucine) in the amino acid sequence of the wild type AtPPO1 was used as a positive control (the amino acid sequence was represented by SEQ ID NO: 9) (Li et al. Development of protoporphyrinogen oxidase as an efficient selection marker for agrobacterium tumefaciens-mediated transformation of maize. Plant physiol. 2003 133:736-747).

The CyPPO variants used in generation of *A. thaliana* transformants were listed in Table 12:

【Table 12】

CyPPO2 mutation	Line No.	CyPPO4 mutation	Line No.	CyPPO8 mutation	Line No.
-----------------	----------	-----------------	----------	-----------------	----------

Y373M	32	Y375M	14	F363M	22
	34		31		51
	38				52
	39			F363V	1
	40				18
Y373V	34			F363L	1
Y373I	23				33
	34			A162L	1
Y373L	23				5
	43			A162C + V308M + F363M	46
Y373C	40				48
V318M+Y373I	1				
	3				
V173S+A175C+Y373M	3				
	4				
	51				
	72				
	84				
A175C+V318M+Y373M	4				
	8				

5-2. Seed germination

Tiafenacil tolerance of *A. thaliana* was confirmed using T₂ seeds which survived under 1 μ M tiafenacil spray at T₁ generation. The transformants were introduced Y373M mutant of CyPPO2, Y375M mutant of CyPPO4, or F363M mutant of CyPPO8 (CyPPO2 Y373M transformant, CyPPO4 Y375M transformant, and CyPPO8 F363M transformant, respectively). Glufosinate tolerance of the transformant

was confirmed by sowing *A. thaliana* seeds in a medium supplemented with glufosinate (50 μ M PPT).

The result was shown in FIG. 32.

No. 32, No. 34, and No. 28 of CyPPO2 Y373M transformants germinated, No. 14 and No. 31 of CyPPO4 Y375M transformants, and No. 22, No. 51 and No. 52 of CyPPO8 F363M transformants germinated even in 1/2 MS media containing 1 μ M or higher concentration of tiafenacil. In addition, the wild type *A. thaliana* (Col-0) seeds used as a negative control did not germinate in 1/2 MS media containing 70 nM tiafenacil, and mutant AtPPO1 transformants (AtPPO1 SLYM) used as a positive control germinated even in 1/2 MS media containing 1 μ M tiafenacil.

5-3. Confirmation of segregation ratio of tolerance trait in T₂ generation

seeds

In order to determine inheritance, segregation ratios were investigated with T₂ seeds. The results were shown in Tables 13 to 15.

【Table 13】

CyPPO2 transformants (T ₂)		
Mutation	Line No.	Segregation ratio
Y373M	32	2.53:1
	34	3.21:1
	38	3.18:1
	39	3.17:1
Y373V	34	3.13:1
Y373I	23	2.70:1
	34	3.55:1
Y373L	5	2.7:1
	23	2.96:1
	43	3.3:1
Y373C	40	3.32:1

V318M+Y373I	1	2.95:1
	3	2.95:1
V173S+A175C+Y373M	3	3.27:1
	4	2.5:1
	72	3.08:1
	84	2.95:1
	51	3.32:1
A175C+V318M+Y373M	4	2.8:1
	8	2.57:1

【Table 14】

CyPPO4 transformants (T ₂)		
Mutation	Line No.	Segregation ratio
Y375M	14	2.53:1
	31	3:1

【Table 15】

CyPPO8 transformants (T ₂)		
Mutation	Line No.	Segregation ratio
F363M	22	3.08:1
	51	3.54:1
	52	3.04:1
F363V	1	2.52:1
	18	2.57:1
F363L	1	2.54:1
	33	2.77:1
A162L	1	2.5:1
	5	3.5:1

A162C + V308M +	46	2.6:1
F363M	48	2.59:1

As shown in Table 13, in case of the CyPPO2 mutant gene inserted transformant, No. 32, No. 34, No. 38, and No. 39 lines of Y373M variant, No. 34 line of Y373V variant, No. 23, and No. 34 lines of Y373I variant, No. 23, and No. 43 lines of Y373L variant, No. 40 line of Y373C variant, No. 1 and No. 3 lines of V318M+Y373I variant, No. 3, No. 4, No. 72, No. 84, and No. 51 lines of V173S+A175C+Y373M variant, and No. 4 and No. 8 lines of A175C+V318M+Y373M variant exhibited approximately 3:1 of tolerance versus susceptible individuals, and thereby it was demonstrated that single copy of transgenes were integrated to *A. thaliana* genome according to Mendel's law.

As shown in Table 14, in case of the CyPPO4 mutant gene inserted transformant, No. 14 and No. 31 lines of Y375M variant exhibited approximately 3:1 of tolerance versus susceptible individuals, and thereby it was demonstrated that single copy of transgenes were integrated to *A. thaliana* genome.

As shown in Table 15, in case of the CyPPO8 mutant gene inserted transformant, No. 22, No. 51 and No. 52 lines of F363M variant, No. 1 and No. 18 lines of F363V variant, No. 1 and No. 33 lines of F363L variant, No. 1 and No. 5 lines of A162L variant, and No. 46 and No. 48 lines of A162C+V308M+F363M exhibited approximately 3:1 of tolerance versus susceptible individuals, and thereby it was demonstrated that single copy of transgenes integrated to the *A. thaliana* genome.

5-4. Investigation of CyPPO protein expression in herbicide-tolerant *A. thaliana* (T₂)

The expression of CyPPO2 Y373M, CyPPO4 Y375M or CyPPO8 F363M in protein level was examined the expression of introduced genes. After approximately 100 mg of *A. thaliana* T₂ transformant leaves were pulverized together with liquid nitrogen, the protein was extracted by adding protein extraction buffer (0.05 M Tris-Cl pH 7.5, 0.1 M NaCl, 0.01 M EDTA, 1% Triton X-100, 1 mM DTT). Western blotting was conducted using the extracted protein. After electrophoresis, the proteins were

transferred to PVDF (polyvinylidene difluoride) membrane, and then western blotting was conducted using anti-HA antibody (Santacruz) and anti-actin antibody (loading control, comparison of the amount of experimental protein; Abcam).

The result was shown in FIG. 33.

5 The level of mutant PPO protein expression was similar in CyPPO2 Y373M transformant and CyPPO8 F363M transformant, whereas CyPPO4 Y375M exhibited low level of expression of PPO variant.

The transformant lines were further progressed by one generation (T_3 generation), and it confirmed that each line of transformants had tolerance to herbicides
10 (refer to the Example 5-5). This indicates that expression of introduced genes is maintained as progressing the generation and they conferred herbicide tolerance.

5-5. Verification of herbicide tolerance of transformed *A. thaliana*

In order to demonstrate the herbicide tolerance of amino acid variant encoding genes of
15 CyPPO2, CyPPO4 and CyPPO8 in a plant, herbicide tolerance was tested to T_2 generation or T_3 generation. All spray tests was performed just before bolting, approximately 4 weeks after transplanting. One hundred milliliters of herbicides with solution (0.05% Silwet L-77) per 40 x 60 cm area (0.24 m^2) were sprayed.

The result observed at the 7th day after spraying to CyPPO2 Y373M variant,
20 CyPPO4 Y375M variant, and CyPPO8 F363M variant were shown in FIG. 34 (the result of tiafenacil treatment), FIG. 35 (the result of saflufenacil treatment), and FIG. 36 (the result of fomesafen treatment). The wild type Col-0 (negative control) died, while AtPPO1 SLYM (positive control) and the experimental groups, No. 38, No. 39 and No. 34 lines of CyPPO2 Y373M transformant, and No. 22, No. 51 and No. 52 lines of
25 CyPPO8 F363M transformant exhibited continuous growth all in tiafenacil 5 μM , saflufenacil 5 μM and fomesafen 5 μM . No. 14 line of CyPPO4 Y375M transformant exhibited continuous growth in saflufenacil 5 μM and fomesafen 5 μM .

Tiafenacil 25 μM or saflufenacil 75 μM was treated to T_3 transformants of Y373C, Y373I, Y373L, Y373M and Y373V mutant genes of CyPPO2. And tiafenacil
30 25 μM or saflufenacil 100 μM was sprayed to T_3 transformant of F360L, F360V and

A162L mutant genes of CyPPO8.

Tiafenacil 5 μ M was sprayed to T₂ transformant of V318M+Y373I, V173S+A175C+Y373M and A175C+V318M+Y373M mutant genes of CyPPO2. Tiafenacil 10 μ M was sprayed to T₂ generation of the transformant of
5 A162C+V308M+F363M mutant gene of CyPPO8.

The result of observed at the 7th day after spraying was shown in Table 16 (Injury index) and FIG. 37 (CyPPO2 variant; T₃ generation (upper, bottom)/T₂ generation (middle)) and FIG. 38 (CyPPO8 variant; T₃ generation (upper, middle)/T₂ generation (bottom)).

10 【Table 16】

Injury index

	Line No.	Tiafenacil	Saflufenacil
	Concentration	5 μ M	5 μ M
Col-0 (wild type)		5	5
CyPPO2		5	NT
CyPPO2 variants (T ₃)		25 μ M	75 μ M
Y373M	40-4	2.5	NT
Y373C	40-3	2	1.5
Y373I	23-2	0	1
	34-2	0	0.75
Y373L	23-9	1.5	1.6
	43-1	1.5	3
Y373V	34-10	2	2
CyPPO2 variants(T ₂)		5 μ M	75 μ M
V318M+Y373I	1	0	NT
	3	0	NT
V173S+A175C+Y373M	3	1.5	NT
	4	2	NT
	51	2	NT

	72	2	NT
	84	3	NT
A175C+V318M+Y373M	4	0	NT
	8	3	NT
CyPPO8		5	5
CyPPO8 variants (T ₃)		5 μ M	75 μ M
F363M	22	0	NT
	51	0	NT
	52	0	NT
		25 μ M	100 μ M
F363L	33-7	1.5	2.5
	1-3	2.5	1.5
F363V	1-7	0.5	0
	18-1	0.5	0
A162L	1-6	3.4	4
	5-4	3.4	3.8
	5-7	3.9	3.8
CyPPO8 variants(T ₂)		10 μ M	100 μ M
A162C + V308M + F363M		46	1
		48	1
NT		NT	NT
CyPPO4 variants		5 μ M	5 μ M
Y375M		14	4
		2	2

NT: Not tested

Injury index of the Table 16 was evaluated by the criteria of the following Table 17 (this was equally applied to injury index provided in the context):

【Table 17】

Injury index	Symptom
0	No damage

1	Dried leaf end
2	Over 20% and less than 30% of the plant was scorched
2.5	Over 30% and less than 50% of the plant was scorched
3	Over 50% and less than 70% of the plant was scorched
4	Over 70% of the plant was scorched
5	The whole plant was dried and died

As shown in Table 16 and FIG. 37, all transformants of CyPPO2 variants respectively grew continuously in not only 5 μ M but also 25 μ M of tiafenacil.

As shown in Table 16 and FIG. 38, transformants of each CyPPO8 variant grew continuously in not only 10 μ M but also 25 μ M of tiafenacil, and grew continuously in saflufenacil 100 μ M.

In addition, the tolerance level of transformants (T_3) in which CyPPO2 Y373I and CyPPO8 F363V were introduced respectively was confirmed in each 50 μ M of herbicides (flumioxazin, sulfentrazone, tiafenacil or saflufenacil). AtPPO1 SLYM was used as the tolerance control group (positive control). Tiafenacil, saflufenacil, flumioxazin, or sulfentrazone were treated at a concentration of 50 μ M respectively, and after 7 days, injury index was evaluated. The result was shown in FIG. 45 and Table 18. For reference, the molecular weight (MW) of tiafenacil is 511.87, and the molecular weight of saflufenacil is 500.85, and the molecular weight of flumioxazin is 354.34, and the molecular weight of sulfentrazone is 387.18. Each herbicide at a concentration of 50 μ M was evenly sprayed with 100ml on a 40 x 60 cm area (0.24 m²). The converted treatment dosages correspond to 106.7 g ai/ha of tiafenacil, 104.4 g ai/ha of saflufenacil, 73.8 g ai/ha of flumioxazin, and 80.7 g ai/ha of sulfentrazone, respectively.

20 【Table 18】

Injury Index			
	AtPPO1	CyPPO8	CyPPO2

	SLYM	F363V	Y373I
Tiafenacil	4	2	2
Saflufenacil	0-1	0-1	0-1
Flumioxazin	4-5	2	3
Sulfentrazone	0-1	0-1	0-1

As shown in FIG. 45 and Table 18, tolerance of mutant gene transformants were similar or higher compared to AtPPO1 SLYM which was known for herbicide tolerance.

While wild type *A. thaliana* died after 0.8 μ M tiafenacil treatment, the PPO mutant transformants exhibited continuous growth in 5 μ M, 10 μ M, or 25 μ M tiafenacil treatment.

From this result, the CyPPO variants are expected to give various PPO-inhibition herbicide tolerances to other plants as well as *A. thaliana*.

10 **5-6. Confirmation of transgene stability during generation passage**

This test is to confirm that genes introduced in *A. thaliana* were stably inherited and expressed even if generation progresses.

The CyPPO2 Y373I mutant and CyPPO8 F363V mutant transformants were developed to T₄, or T₅ generation as follow; T₃ lines 23-2, 23-7, 34-2 of CyPPO2 Y373I and 1-7, 18-1, 18-7 of CyPPO8 F363V. The herbicide tolerance was maintained through T₃ to T₅ generation of each line indicating the stability of transgene during generations.

Specifically, 15 μ M tiafenacil or 150 μ M saflufenacil was treated to T₄ or T₅ lines, and 7 days after herbicide treatment, damage level was evaluated. The result was shown in FIG. 46 (T₄ line) and FIG. 47 (T₅ line).

20 In addition, western blotting was conducted to confirm protein expression. The proteins were extracted from transformants. After grinding seedling using liquid nitrogen, protein extraction buffer (0.05 M Tris-Cl pH 7.5, 0.1 M NaCl, 0.01 M EDTA, 1% Triton X-100, 1 mM DTT) was added and the total protein was extracted. The extracted protein was transferred to PVDF membrane after electrophoresis, western

blotting was conducted using anti-HA antibody (Santacruz). The expressed proteins in the transformant were detected. The result was shown in FIG. 48 (T₄ line) and FIG. 49 (T₅ line).

As shown in FIG. 46 to FIG. 49, herbicide tolerance of all the generations of T₄ and T₅ was maintained, and PPO protein expression was also confirmed. Specifically, while Col-0 (negative control) completely died when 15 μ M tiafenacil or 150 μ M saflufenacil was treated, T₄ and T₅ transformants were maintained without injury (Injury index 0). For reference, when 25 μ M tiafenacil or 75 μ M saflufenacil was treated in T₃ generation, the level of injury index was 0~1.

Example 6. Generation of rice transformants using CyPPO variants and PPO-inhibiting herbicide tolerance test

6-1. Construction of rice transformation vector and generation of rice transformants

A binary vector having ORF of Bar gene (glufosinate-tolerant gene) and ORF of each amino acid mutant gene of CyPPO2 or CyPPO8 was constructed and used for rice transformation. Each gene were cloned in pCAMBIA3301 vector (refer to FIG. 39). The CaMV 35S promoter for expression of Bar gene and the 35S terminator for transcription termination were used.

In order to express mutant genes of CyPPO2 or CyPPO8 in a plant, the ubiquitin promoter of a corn and the NOS terminator were used. In addition, the transit peptide (TP) of AtPPO1 was fused in the N-terminal region of CyPPO2 or CyPPO8 mutant. Each vector constructed was transformed to *Agrobacterium tumefaciens* LBA4404 competent cell (Takara) by an electric shock method.

The transformed *Agrobacterium* was used to transform Dongjin rice (wild type cultivar). After removing seed husk of Dongjin rice, disinfecting, and dark culturing in N6D media at 32 °C for 5 days, rice seeds were mixed with the transformed *Agrobacterium* solution, and then transplanted on 2N6-AS100 media. It was incubated at 28 °C for 1 day in dark, and then incubated at 23.5 °C for 4 days in dark. Seeds were incubated on N6D cf500 ppt4 media supplemented with appropriate concentration of

phosphinothricin (Duchefa) at 28 °C for 10 days under light, and transformed callus was selected among infected seeds. The selected callus was moved on REIII cf500 ppt4 media supplemented with appropriate concentration of phosphinothricin (Duchefa), thereby inducing a plant.

5 The composition of the used media was listed in Table 19:

【Table 19】

Media name	Ingredient	Usage
N6D	N6 powder	4 g
	Sucrose	30 g
	L-Proline	2.878 g
	Casamino acid	0.3 g
	Myo-Inositol	0.1 g
	2,4-Dichlorophenoxy (dissolve in ethanol)	2 mg
	Phytigel	4 g
	Distilled Water up to	1L (pH 5.8)
2N6-AS100	N6 powder	4 g
	Sucrose	30 g
	D-glucose (Monohydrate)	10 g
	Casamino acid	0.3 g
	2,4-Dichlorophenoxy (dissolve in ethanol)	2 mg
	Phytigel	4 g
	Acetosyringone (20 mg/ml, dissolve in DMSO)	1 ml
	Distilled Water up to	1L (pH 5.2)
N6D cf500 ppt4	N6 powder	4 g
	Sucrose	30 g
	L-proline	2.878 g
	Casamino acid	0.3 g
	Myo-inositol	0.1 g
	2,4-Dichlorophenoxy (2 mg/ml in ethanol)	1 ml

	Phytigel	4 g
	Cefotaxime sodium	500 mg
	Distilled Water up to	1L (pH 5.8)
REIII cf500 ppt4	MS vitamin powder	4.41 g
	Sucrose	30 g
	Sorbitol	30 g
	Casamino acid	2 g
	NAA (1mg/ml)	20 μ l
	Kinetin	2 mg
	Myo-inositol	0.1 g
	Phytigel	4 g
	Cefotaxime sodium	500 mg
	Distilled Water up to	1L (pH 5.8)

6-2. Verification of PPO-inhibiting herbicide tolerance of transformed rice

In order to examine the level of herbicide tolerance of transformants of CyPPO2 and CyPPO8 mutant genes in a monocotyledon crop, herbicide tolerance was tested in T₀ generation plant of Dongjin rice in which each gene was transformed.

After taking tillers of 5-week-old rice of T₀ generation of CyPPO2 Y373M transformant and CyPPO8 F363M transformant, they were allowed to grow, and tiafenacil or saflufenacil were treated, respectively. Two hundreds milliliters of 200 μ M tiafenacil (corresponding to 853 g ai/ha) or 500 μ M saflufenacil (corresponding to 2,085 g ai/ha) was treated in the area of 40 X 60 cm. For reference, the recommended treatment dosage of tiafenacil for weed control is approximately 150 g ai/ha, and the recommended treatment dosage of saflufenacil for weed control is under 145 g ai/ha.

T₂ transformants of CyPPO2 Y373M and CyPPO8 F363M were used for herbicide treatment at 47th day after sowing. One hundred milliliters of 200 μ M tiafenacil (corresponding to 420 g ai/ha) and 400 μ M saflufenacil (corresponding to 840 g ai/ha) were sprayed respectively in the area of 40 X 60 cm. The result observed at the 7th day after spraying was shown in FIG. 40 (the result showing T₀ generation plants of

CyPPO2 Y373M transformant line No. 1 and No. 3 and CyPPO8 F363M transformant line No. 2 and No. 3 after tiafenacil or saflufenacil treatment) and FIG. 41 (showing T₂ generation plants of CyPPO2 Y373M transformant line #3 and CyPPO8 F363M transformant line #3 after tiafenacil or saflufenacil treatment), respectively. “non-GM”
5 in FIG. 40 indicates non-transformed wild type Dongjin rice, and was used as a negative control.

As shown in FIG. 40 and FIG. 41, severe damages were in the negative control, wild type Dongjin rice, after treatment of tiafenacil or saflufenacil, but no or weak damage was observed in T₀ generation plants of CyPPO2 Y373M transformant
10 (represented by “CyPPO2YM”) line No. 1 and No. 3 and T₂ generation plants of CyPPO2 Y373M transformant #3-1. No damage was observed in T₀ generation plants of CyPPO8 F363M transformant (represented by “CyPPO8FM”) line #1 and #3 and T₂ generation plants of CyPPO8 F363M transformant #3-1 compared to the wild type Dongjin rice after treatment of tiafenacil or saflufenacil.

15 All CyPPO2 Y373M transformant and CyPPO8 F363M transformant rice plants exhibited tolerance after the treatment of tiafenacil or saflufenacil of higher concentration than the recommended concentration for weed control. This indicated that the genes introduced to rice plants were inherited and expressed stably, showing PPO-inhibiting herbicide tolerance through generations.

20

6-3. Verification of protein expression in rice transformants

In order to confirm that mutant protein (CyPPO2 Y373M or CyPPO8 F363M) was expressed in CyPPO2 Y373M transformant and CyPPO8 F363M transformant, the protein was extracted in a leaf from each transformant line, and western blotting was
25 conducted.

For this, 5 ml/g tissue of extraction buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40) and protease inhibitor (Xpert Protease Inhibitor Cocktail Solution, GenDepot) were added to ground leaf tissue of each transformant. The extracted protein was loaded to SDS-PAGE gel, and transferred to PVDF
30 membrane. The membrane was incubated with CyPPO2 or CyPPO8 peptide-specific

primary antibody (GenScript) at the ratio of 1:1000. Protein was detected by HRP-conjugated secondary antibody using ECL reagent and Luminograph (Atto).

The obtained images were shown in FIG. 42 (CyPPO2 Y373M expression) and FIG. 43 (CyPPO8 F363M). CyPPO protein was detected in CyPPO2 Y373M transformant line #1 and #3 and CyPPO8 F363M transformant line #2 and #3.

6-4. Copy number analysis of introduced genes in transformed rice

The genomic DNA was extracted from leaf tissues of CyPPO8 F363M transformant to analyze the copy number of the transgene.

Genomic DNA was extracted as follows. After grinding leaf tissue of the transformed rice using a pestle and a mortar in liquid nitrogen, 5 ml/g tissue of DNA isolation buffer (2% (w/v) CTAB, 1.5 M NaCl, 25 mM EDTA, 0.2% (v/v) beta-mercaptoethanol, 100 mM Tris-Cl (pH 8.0)) was added and vortexed. After heating at 60 °C for over 1 hour, 1 volume of chloroform:isoamyl alcohol (24:1) was added and mixed with inverting. After centrifugation at the condition of 7000 xg for 10 minutes at 4 °C, supernatant was moved to a new tube, and 2.5 volume of ethanol was mixed. After centrifugation at 5000 xg for 5 minutes at 4 °C, supernatant was discarded and the pellet was dissolved in TE buffer (LPSS). After adding 20 µg/ml RNase A (Bioneer), it was incubated at 37 °C for 30 minutes. After adding 1 volume of phenol:chloroform (1:1), it was mixed and centrifuged at 10,000 xg for 10 minutes at 4 °C. Supernatant was moved to a new tube, and then 1 volume of chloroform:isoamyl alcohol (24:1) was added and mixed. After centrifugation at 10,000 xg for 10 minutes for 4 °C, supernatant was moved to a new tube, and 0.1 volume of NaOAc (pH 5.2) and 2 volume of ethanol were added and mixed. Then, it was centrifuged at 5,000 xg for 5 minutes at 4 °C, and the pellet was washed with 70% ethanol. After air dry, genomic DNA was dissolved in an appropriate amount of TE buffer.

The 10~40 µg of extracted DNA was digested for overnight using EcoRI (Enzynomics).

Then, after 0.8% (w/v) Agarose gel electrophoresis (50 V), gel was treated as follows:

- 1) depurination: 0.25 N HCl, 15 minutes shaking
- 2) denaturation: 0.5 M NaOH, 1.5 M NaCl, 30 minutes shaking
- 3) neutralization: 0.5 M Tris (pH 7.5), 1.5 M NaCl, 20 minutes shaking

Thereafter, DNA fragments were moved to nitrocellulose membrane (GE healthcare) using a capillary transfer method, cross linking was performed using UV Crosslinker (UVC-508; ULTRA LUM Inc.).

Hybridization was performed by the following method: The nitrocellulose membrane was dipped in Easyhybridization solution (Roche), and incubated at 42 °C for 3 hrs. Then, the solution was discarded, substituted with a new DIG Easyhybridization solution, and incubated for overnight at 42 °C.

The probe (DIG-labeled CyPPO8-M probe) was labelled by PCR reaction as follows:

Materials

	Template (CyPPO8 plasmid DNA)	0.5μl
15	10X buffer	3μl
	DIG-dNTP	2μl
	forward primer (10μM)	3μl
	reverse primer (10μM)	3μl
	DDW	18μl
20	e-Taq polymerase (Solgent Inc.)	0.5μl
		total 30μl

【Table 20】

Conditions for PCR reaction

94°C	3 min	35cycles
94°C	30 sec	
58°C	30 sec	
72°C	1 min	
72°C	5 min	

Sequences for primers

Forward primer for CyPPO8 F363M probe: GCGTTAACGGGTGCATTAGGC (SEQ ID NO: 158)

Reverse primer for CyPPO8 F363M probe: TGGAAAGAGTGTTGAACTCC (SEQ ID

NO: 159)

After PCR product was electrophoresed in agarose gel, the labelled probe band was extracted.

After hybridization with labelled probe, membrane was washed in low stringency washing buffer (2X SSC, 0.1% SDS) followed by high stringency washing buffer (0.5X SSC, 0.1% SDS). Southern blotting signal was detected as follows:

1) shaking for 30 minutes after adding blocking buffer (Roche) to the membrane

2) shaking for 30 minutes after adding DIG antibody (anti-digoxigenin-AP Fab fragments, Roche)

3) shaking for 15 minutes in washing buffer (Roche)

4) shaking for 3 minutes after adding detection buffer (Roche)

5) After applying CDP-Star, ready-to-use (Roche) on the membrane, developing the blot on x-ray film.

The result was shown in FIG. 44. CyPPO8 F363M #3 line showed one band, which indicates single copy of the gene was inserted in rice genome.

Example 7. Generation of soybean transformants using CyPPO variants and PPO-inhibiting herbicides tolerance test

7-1. Generation of soybean transformants

Vectors to transform soybean plant with CyPPO2 Y373M or CyPPO8 F363M gene were constructed.

The sequence of transit peptide of *A. thaliana* PPO1 was fused to 5' region of CyPPO2 Y373M or CyPPO8 F363M (refer to FIG. 31), and the fused gene was amplified and isolated for vector construction.

PCR reaction mixture (total 50 µl) was prepared by mixing 1 µl of template (the vector which was used for *A. thaliana* transformation), 5 µl of 10X buffer, 1 µl of dNTP mixture (each 10 mM), 1 µl of TOPO-cTP_F primer (10 µM), 1 µl of TOPO-CyPPO2_R or TOPO-CyPPO8_R primer (10 µM), 40 µl of DDW, and 1 µl of Pfu-X (Solgent, 2.5 unit/µl), and amplification was performed under conditions of 1 cycle of

94 °C for 4 minutes, and 25 cycles of 94 °C for 30 seconds, 56 °C for 30 seconds and 72 °C for 1.5 minutes, and 1 cycle of 72 °C for 5 minutes.

Primers used were summarized in Table 21:

【Table 21】

Primer	Sequence
TOPO-cTP F	CAC CAT GGA GTT ATC TCT TC (SEQ ID NO: 160)
TOPO-CyPPO2 R	TCA GAT CGA TCG AGT ATC TG (SEQ ID NO: 161)
TOPO-CyPPO8 R	TTA ACC CAA ATA ATC TAA CA (SEQ ID NO: 162)

5 Each amplified product was ligated into pENTR-TOPO vector (Invitrogen, refer to FIG. 50) using pENTR Directional TOPO cloning kits (Invitrogen), and the ligation product was transformed to DH5 alpha competent cell (Invitrogen).

Cloned gene in pENTR-TOPO vector was transferred to pB2GW7.0 binary vector to transform plants. Gateway LR Clonase II Enzyme Mix kit (Invitrogen) was used for pB2GW7.0 vector construction. After mixing pENTR/D-TOPO vector in
10 which CyPPO2YM or CyPPO8FM gene was inserted, TE buffer, and LR Clonase II enzyme mix, the mixture was incubated at 25 °C for 1 hr. After Proteinase K solution (Invitrogen) was added to the reaction mixture, it was incubated at 37 °C for 10 minutes, and it was transformed to DH5 alpha competent cell.

15 *Agrobacterium tumefaciens* EHA105 (Hood et al., New Agrobacterium helper plasmids for gene transfer to plants (EHA105). Trans Res. 1993 2:208-218) was electro-transformed with the binary vector constructs above. 'Kwangan' soybean plant was used for transformation. After removing seed coat from soybean seed, hypocotyl was cut and wounded 7-8 times by surgical scalpel (#11 blade). Approximately 50 pieces of
20 explants were mixed with transformed Agrobacterium EHA105, and the mixture was sonicated for 20 seconds and then incubated for 30 minutes for inoculation. It was placed on CCM (Co-cultivation media; 0.32 g/L Gamborg B5, 4.26 g/L MES, 30 g/L sucrose, 0.7% agar) media. Then, it was co-cultured in a growth chamber (25 °C, 18 hr light/ 6 hr dark) for 5 days.

25 Then it was washed for 10 minutes in liquid 1/2 SIM (shoot induction media; 3.2 g/L B5 salt, 1.67 mg/L BA, 3 mM MES, 0.8% (w/v) agar, 3% (w/v) sucrose, 250

mg/L cefotaxime, 50 mg/L vancomycin, 100 mg/L ticarcillin, pH 5.6) and was placed on SIM without antibiotics and cultured in a growth chamber (25 °C, 18 hr light/ 6 hr dark) for 2 weeks.

The shoot-induced explants were transplanted on SIM-1 (SIM media supplemented with 10 mg/L DL-phosphinothricin, pH 5.6). The browned shoots were transplanted on SEM (shoot elongation media; 4.4 g/L MS salt, 3 mM MES, 0.5 mg/L GA3, 50 mg/L asparagine, 100 mg/L pyroglutamic acid, 0.1 mg/L IAA, 1 mg/L zeatin, 3% (w/v) sucrose, 0.8% (w/v) agar, 250 mg/L cefotaxime, 50 mg/L vancomycin, 100 mg/L ticarcillin, 5 mg/L DL-phosphinothricin, pH 5.6). The elongated shoots over 4 cm height were transferred on RIM (root induction medium; 4.4 g/L MS salt, 3 mM MES, 3% sucrose, 0.8% agar, 50 mg/L cefotaxime, 50 mg/L vancomycin, 50 mg/L ticarcillin, 25 mg/L asparagine, 25 mg/L pyroglutamic acid, pH 5.6).

When the roots grew sufficiently, the plants were moved to bed soil (Bioplug No. 2, Farmhannong) mixed with vermiculite in 2:1 (v/v). After 10 days, leaves were painted with 100 mg/L DL-phosphinothricin.

7-2. Analysis of introduced genes in soybean transformants

In order to analyze the copy number of introduced genes in CyPPO2 Y373M transformed soybean T₀ plants (line no. 12, 14, 16, 24, 25, 27, 28, 34, and 41) and CyPPO8 F363M transformed soybean T₁ plant (line no. 3, 5, 7, 9, 11, 14, 17, 36, and 44), genomic DNA was extracted with 250 mg of leaf tissue of each transformed plant referring to Example 6-4.

The 10~40 µg of genomic DNA was digested with EcoRI (Enzynomics) for overnight, and southern blotting was performed following the method in Example 6-4.

The Bar DNA probe for hybridization was prepared by labelling with DIG (Digoxigenin)-dNTP by PCR reaction. PCR reaction mixture (total 50 µl) was prepared by mixing 0.5 µl of template (the vector which was used for soybean transformation), 5 µl of 10X buffer, 10 µl of DIG-dNTP mixture (dATP, dCTP, dGTP, respectively, 0.5 mM, dTTP 0.32 mM, DIG-11-dUTP 0.18 mM), 0.5 µl of forward primer (100 µM), 0.5 µl of reverse primer (100 µM), 33 µl of DDW, and 0.5 µl of e-Taq (Solgent, 2.5 unit/µl),

and amplification was performed under conditions of 1 cycle of 94 °C for 4 minutes, and 35 cycles of 94 °C for 30 seconds, 56 °C for 30 seconds and 72 °C for 30 seconds, and 1 cycle of 72 °C for 5 minutes.

Bar probe primer:

5 Forward primer for bar probe: 5'- TTC CGT ACC GAG CCG CAG GA -3'
(SEQ ID NO: 163)

Reverse primer for bar probe: 5 '- CGT TGG GCA GCC CGA TGA CA -3'
(SEQ ID NO: 164)

For comparison, the genomic DNA of non-transformed Kwangan soybean (WT)
10 was used as a negative control.

The result was shown in FIG. 51. The number of bands shown on the film in FIG. 51 means the number of inserted genes. In case of non-transformed Kwangan soybean (WT), no band was detected, and it was demonstrated that one copy of CyPPO gene was integrated into the genome of Kwangan soybean in CyPPO2 Y373M
15 transformant lines No.14, 25, 34, and 41 and in CyPPO8 F363M transformant lines No. 3, 5, 7, 11, 14, and 17.

Hereinafter, using the single copy inserted transformant lines, herbicide tolerance was evaluated and CyPPO protein expression was tested.

20 **7-3. Verification of herbicide tolerance of transformed soybeans**

In order to examine the level of herbicide tolerance of CyPPO2 Y373M or CyPPO8 F363M transformed soybean plants, herbicide was applied to soybean transformants (T₂ generation).

Twenty micromolar of tiafenacil (corresponding to 42 g ai/ha) or 150 μM
25 (corresponding to 315 g ai/ha) or 300 μM (corresponding to 630 g ai/ha) of saflufenacil was sprayed respectively to the soybean of V2~3 stage. The 100 ml herbicide of the concentration above was evenly sprayed on the area of 40 X 60 cm, and after 5 days, herbicide tolerance was evaluated. Non-transformed soybean (represented by Kwangan soybean) was used as a control.

30 FIG. 52 shows the result. While non-transformed Kwangan soybean died at

each herbicide treatment with injury index 9, the injury index of CyPPO2 Y373M or CyPPO8 F363M transformants was 1~3 when treated with 20 μ M tiafenacil. And the injury index of CyPPO2 Y373M transformants was 0 when treated with 150 μ M saflufenacil and that of CyPPO8 F363M transformants was 0 when treated with 300 μ M

5 saflufenacil. The injury index was determined by the following criteria:

【Table 22】

Injury Index	Damage description by herbicide treatment
0	No damage
1	Very weak level, very small part of leaves was damaged or chlorosis was observed.
2	Weak damage symptom with a little severe chlorosis, no effect on the whole growth condition
3	No effect on the primary leaf and growing point, a little severe damage was observed in the secondary leaf tissue
4	The whole plant shape was little changed. There was no effect on stem, but chlorosis and necrosis were observed in the secondary growing point and leaf tissues. Considered that re-growth is possible within one week.
5	The whole plant shape was definitely changed. Chlorosis and necrosis were observed in many leaves and growing point. The primary growing point was not damaged, and stem had green color. Considered that re-growth is possible within one week.
6	Strong damage on the growth of a newly growing small leaf was observed. Considered that the plant could survive by the growth in the different growing point. Chlorosis and necrosis were observed in most of leaves, and stem had green color. Considered that re-growth is possible, but damage symptoms were severely observed.
7	Chlorosis was exhibited in most of growing points. Re-growth in one of growing point could be possible, and two leaves had green color partially. Partial chlorosis, necrosis, and green color. The rest part of plants including stem exhibited necrosis.
8	All the growing points were necrosed, and plants are likely to die. One leaf had green color partially.
9	Plant necrosed.

The result shows that while the wild type Kwangan soybean died with herbicide treatment at each concentration, the transformants exhibited weak damage with tiafenacil treatment and hardly exhibited damage with saflufenacil treatment. As a reference, IC₅₀ value for tiafenacil or saflufenacil of CyPPO2 Y373M was 250 nM or 5,000 nM, respectively, and IC₅₀ value for tiafenacil or saflufenacil of CyPPO8 F363M was 183 nM or 5,000 nM, respectively (Tables 10 to 11).

7-4. Verification of protein expression in transformed soybeans

The expression of CyPPO2 Y373M protein or CyPPO8 F363M protein in transformed plants was examined.

In order to examine protein expression, the total protein was extracted in each transformant (T₁) and western blot analysis was conducted. After triturating the leaf tissue of each transformant with liquid nitrogen, protein extraction buffer (0.8% SDS, 4% glycerol, 2% beta-mercaptoethanol, 0.0008% bromophenol blue, 0.125M Tris-Cl, pH 7.4) was added and the total protein was extracted.

The extracted protein was electrophoresed in SDS-PAGE gel and transferred to PVDF membrane. The membrane was labeled with antibody specific to each inserted protein (CyPPO2-specific antibody for CyPPO2 Y373M transformant, CyPPO8-specific antibody for CyPPO8 F363M transformant; GenScript).

The result demonstrated that CyPPO2 Y373M protein or CyPPO8 F363M protein was expressed in all transformant individuals (FIG 53). It indicates that herbicide tolerance of the transformants was conferred by the expression of CyPPO2 Y373M protein or CyPPO8 F363M protein.

Example 8. Generation of rapeseed transformants using CyPPO variants and PPO-inhibiting herbicides tolerance test

8-1. Generation of rapeseed transformants

The vector, in which CyPPO8 F363M gene was inserted, constructed in the Example 5-1 was used to generate rapeseed transformants.

The seeds of rapeseed were sterilized with 70% (v/v) ethanol for 4 minutes and

then with 1.3% (v/v) sodium hypochlorite acid for 30 minutes. After washing 5 times with sterile water, moisture was removed on a sterile filter paper, and the seeds were transplanted to MSO media (4.43 g/L MS salt, 30 g/L sucrose, 3 g/L phytagel, pH 5.8), and cultured for 5 days in a culture room at 25±1°C under the light condition (16 h light / 8 h dark, 25,000 Lux).

After *Agrobacterium* was transformed with the vector inserted with CyPPO8 F363M gene, it was inoculated in LB media supplemented with spectinomycin (100 mg/L) and rifampicin (50 mg/L), and cultured for over 16 hrs in a shaking incubator at 28 °C. Cotyledons and hypocotyls of a rapeseed plant were cut and co-cultured with the *Agrobacterium* for 3 days (dark condition, 25±1°C). The inoculated plant tissues were transferred to a selection media (4.43 g/L MS salt, 20 g/L sucrose, 0.2 mg/L NAA, 8 µM TDZ, 0.01 mg/L GA3, 50 µM silver thiosulfate, 10 mg/L PPT, 500 mg/L carbenicillin, 4 g/L phytagel, pH 5.8) and cultured in a growth chamber (25±1 °C, 16 h light/8 h dark).

The inoculated plant tissues were subcultured every two weeks and then redifferentiated shoots from callus were transferred to root induction media (4.43 g/L MS salt, 30 g/L sucrose, 3 g/L phytagel, activated charcoal 3 g/L, pH 5.8), thereby inducing roots. Root-induced small plants were transferred to pots, and then transformants were confirmed using excised leaves.

8-2. Verification of herbicide tolerance of transformants

After seeds of the rapeseed transformants (T₁) and Youngsan rapeseed (wild type rapeseed, control) were sterilized with 50% (v/v) sodium hypochlorite for 30 minutes, they were washed 5 times with sterile water. Transformants were sown in a selection media (1/2 MS, 50 nM tiafenacil, pH 5.8) and the wild type rapeseeds were sown in 1/2 MS media. Individuals survived after 7 days were transplanted to pots and cultured in a growth chamber (25±1°C, 16 h light/8 h dark). After 35 days of transplantation, 10 µM tiafenacil and 10 µM saflufenacil were treated respectively by 100 mL (0.05% Silwet L-77) per a tray (40 x 60 cm, 0.24 m²) in which the pot placed.

The results of 7 days after treatment of the herbicide were shown in FIG. 54

(tiafenacil treatment) and FIG. 55 (saflufenacil treatment). In FIG. 54 and FIG. 55, “Youngsan” represented the wild type Youngsan rapeseed. The injury level was shown in Table 23 by evaluating with injury index 0-5 (no damage-death; refer to Table. 17):

【Table 23】

	10 μ M Tiafenacil	10 μ M Saflufenacil
WT (Youngsan)	4	4
CyPPO8 F363M 2-4	2	NT
CyPPO8 F363M 2-7	2	NT
CyPPO8 F363M 2-8	1	NT
CyPPO8 F363M 2-6	NT	0
CyPPO8 F363M 2-9	NT	0
CyPPO8 F363M 2-10	NT	0

5 (NT: Not tested)

While Youngsan (wild type rapeseed, control) exhibited injury level 4, CyPPO8 F363M-2 lines exhibited tolerance injury level 1-2 to 10 μ M tiafenacil (FIG. 54). In addition, after 10 μ M saflufenacil treatment, CyPPO8 F363M-2 lines exhibited no damage (FIG. 55).

10

8-3. Verification of protein expression of transformed rapeseed

The expression of inserted genes in CyPPO8 F363M rapeseed transformants was confirmed. Among CyPPO8 F363M lines, 5 individuals (2-1, 2-2, 2-6, 2-9, and 2-10) confirmed to have herbicide tolerance were selected for western blotting.

15 After approximately 200 mg of leaves of each rapeseed transformant was ground using liquid nitrogen and a pestle, 0.8 mL phenol (Tris-buffered, pH 8.0) and 0.8 mL dense SDS buffer (30% (w/v) sucrose, 2% (w/v) SDS, 0.1 M Tris-Cl, pH 8.0, 5% (v/v) beta-mercaptoethanol) were added and mixed by vortexing for 30 seconds, and centrifugation was conducted at 10,000 xg for 3 minutes. Supernatant was transferred to
20 a new tube, and 5-volume of methanol (4 °C, 0.1 M ammonium acetate) was added and mixed, and then left at -20°C for 30 minutes. Proteins were precipitated by centrifugation at 10,000 xg for 5 minutes, and washed with 1 mL methanol (4 °C, 0.1 M

ammonium acetate) twice and 80% acetone (4 °C) twice, and dried. The dried proteins were dissolved with 2% (w/v) SDS buffer (50 mM Tris-Cl, pH 6.8, 1 mM DTT).

Since the CyPPO8 F363M vector was constructed with HA tag (refer to Example 5-1), western blotting was conducted with HA antibody (Santa Cruz) to
5 confirm the CyPPO8 F363M protein expression.

The western blot result was shown in the top of FIG. 56. It was demonstrated that the CyPPO8 protein was expressed in all individuals except Youngsan (wild type rapeseed, control). Such result indicated that herbicide tolerance was conferred by protein expression of introduced genes.

10 Additionally, the Coomassie blue staining result of the PVDF membrane was shown in the bottom of FIG. 56. It was demonstrated that the amount of protein of each sample was almost equal.

【Claims】

【Claim 1】

A polypeptide selected from the followings:

a polypeptide comprising an amino acid sequence wherein one or more selected
 5 from the group consisting of S63, P91, R92, F169, V173, A175, E228, L229, P316, V318, F337, L340, G351, T352, I353, and Y373 of the amino acid sequence of SEQ ID NO: 1 are independently deleted or substituted with an amino acid which is selected from the group consisting of M(Met), V(Val), I(Ile), T(Thr), L(Leu), C(Cys), A(Ala), S(Ser), F(Phe), P(Pro), W(Trp), N(Asn), Q(Gln), G(Gly), Y(Tyr), D(Asp), E(Glu),
 10 R(Arg), H(His), and K(Lys), and is different from the amino acid at the corresponding position in the wild type;

a polypeptide comprising an amino acid sequence wherein one or more selected from the group consisting of N63, S64, S66, P67, P92, R93, F170, S172, G173, V174, Y175, A176, R190, E230, L231, P318, V320, F339, G340, N341, L342, L352,
 15 G353, T354, I355, Y375, I424, V458, and R465 of the amino acid sequence of SEQ ID NO: 3 are independently deleted or substituted with an amino acid which is selected from the group consisting of M(Met), V(Val), I(Ile), T(Thr), L(Leu), C(Cys), A(Ala), S(Ser), F(Phe), P(Pro), W(Trp), N(Asn), Q(Gln), G(Gly), Y(Tyr), D(Asp), E(Glu), R(Arg), H(His), and K(Lys), and is different from the amino acid at the corresponding
 20 position in the wild type;

a polypeptide comprising an amino acid sequence wherein one or more selected from the group consisting of P84, R85, F156, V160, A162, Q179, P306, V308, F327, L330, I343, N362, and F363 of the amino acid sequence of SEQ ID NO: 5 are independently deleted or substituted with an amino acid which is selected from the
 25 group consisting of M(Met), V(Val), I(Ile), T(Thr), L(Leu), C(Cys), A(Ala), S(Ser), F(Phe), P(Pro), W(Trp), N(Asn), Q(Gln), G(Gly), Y(Tyr), D(Asp), E(Glu), R(Arg), H(His), and K(Lys), and is different from the amino acid at the corresponding position in the wild type; and

a polypeptide comprising an amino acid sequence having 95% or higher
 30 homology with the amino acid sequence of said polypeptides.

【Claim 2】

The polypeptide of claim 1, which is selected from the group consisting of:

a polypeptide comprising an amino acid sequence wherein one or more selected from the group consisting of S63, P91, R92, F169, V173, A175, E228, L229, P316, V318, F337, L340, G351, T352, I353, and Y373 of the amino acid sequence of SEQ ID NO: 1 are independently substituted with an amino acid which is selected from the group consisting of M(Met), V(Val), I(Ile), T(Thr), L(Leu), C(Cys), A(Ala), S(Ser), and F(Phe);

a polypeptide comprising an amino acid sequence wherein one or more selected from the group consisting of A176, P318, V320, and Y375 of the amino acid sequence of SEQ ID NO: 3 are independently substituted with an amino acid which is selected from the group consisting of M(Met), V(Val), I(Ile), T(Thr), L(Leu), C(Cys), and A(Ala);

a polypeptide comprising an amino acid sequence wherein one or more kinds selected from the group consisting of P84, R85, F156, V160, A162, Q179, P306, V308, F327, L330, I343, N362, and F363 among the amino acid sequence of SEQ ID NO: 5 are independently substituted with an amino acid which is selected from the group consisting of M(Met), V(Val), I(Ile), T(Thr), L(Leu), C(Cys), A(Ala), S(Ser), H(His), and G(Gly); and

a polypeptide comprising an amino acid sequence having 95% or higher homology with the amino acid sequence of said polypeptides.

【Claim 3】

The polypeptide of claim 1, which is selected from the group consisting of:

a polypeptide comprising an amino acid sequence comprising one or more amino acid mutations selected from the group consisting of Y373V, Y373I, Y373T, Y373L, Y373C, Y373M, A175C, A175L, A175I, P316A, P316L, V318M, V318T, V318L, G351A, S63T, P91L, R92A, V173S, V173C, V173T, V173L, F169A, E228A, L229F, F337V, L340T, L340I, L340V, T352V, I353T, I353L, I353V, and I353C in the amino acid sequence of SEQ ID NO: 1;

a polypeptide comprising an amino acid sequence comprising one or more

amino acid mutations selected from the group consisting of Y375M, Y375V, Y375I, Y375T, Y375C, A176C, A176L, P318L, V320L, V320M, and P318A in the amino acid sequence of SEQ ID NO: 3;

a polypeptide comprising an amino acid sequence comprising one or more amino acid mutations selected from the group consisting of P84L, R85A, R85C, R85H, R85L, R85T, R85V, F363M, F363V, F363L, F363C, F363I, F363T, A162C, A162L, P306L, P306A, V308L, V308M, N362S, V160S, V160C, I343V, I343T, F156A, Q179G, F327V, and L330T in the amino acid sequence of SEQ ID NO: 5; and

a polypeptide comprising an amino acid sequence having 95% or higher homology with the amino acid sequence of said polypeptides.

【Claim 4】

The polypeptide of claim 3, which is selected from the group consisting of:

a polypeptide comprising an amino acid sequence comprising amino acid mutation of Y373M, Y373V, Y373I, Y373T, Y373L, Y373C, F169A, A175C, A175L, A175I, P316A, P316L, V318M, V318T, V318L, G351A, S63T, P91L, R92A, V173S, V173C, V173T, V173L, E228A, L229F, F337V, L340T, L340I, L340V, T352V, I353T, I353L, I353V, I353C, S63T+Y373M, R92A+Y373M, V173S+Y373M, V173S+Y373I, V173S+Y373L, V173S+Y373V, V173C+Y373M, V173C+Y373I, V173T+Y373M, V173T+Y373I, V173L+Y373M, A175L+Y373M, A175L+Y373I, A175C+Y373M, A175C+Y373I, A175I+Y373M, E228A+Y373M, L229F+Y373T, V318M+Y373M, V318M+Y373I, V318M+Y373V, V318T+Y373I, L340I+Y373M, L340I+Y373I, L340V+Y373M, G351A+Y373M, I353T+Y373M, I353T+Y373I, I353T+Y373L, I353L+Y373M, I353V+Y373M, I353C+Y373M, S63T+V173S+Y373M, S36T+V173S+Y373I, S63T+I353T+Y373M, S63T+I353T+Y373I, V173S+V318M+Y373M, V173T+L340I+Y373M, V173S+A175C+Y373M, A175C+V318M+Y373M, A175L+V318M+Y373M, A175C+I353L+Y373M, A175C+I353V+Y373M, P316A+V318L, P316L+V318L, F337V+Y373M, T352V+Y373M, G351A+T352V+Y373M, or P91L+Y373M, in the amino acid sequence of SEQ ID NO: 1;

a polypeptide comprising an amino acid sequence comprising amino acid

mutation of Y375M, Y375V, Y375I, Y375T, Y375C, A176C, A176L, P318L+V320L, V320M, or P318A+V320L, in the amino acid sequence of SEQ ID NO: 3;

a polypeptide comprising an amino acid sequence comprising amino acid mutation of P84L, R85A, R85C, R85H, R85L, R85T, R85V, F363M, F363V, F363L, F363C, F363I, F363T, A162C, A162L, P306L, P306A, V308L, V308M, N362S, V160S, V160C, I343V, I343T, F156A, Q179G, F327V, L330T, P84L+F363M, R85A+F363M, R85A+F363I, R85C+F363M, R85H+F363M, R85L+F363M, R85T+F363M, R85V+F363M, R85A+A162L+F363M, R85A+A162L+F363I, R85A+A162C+F363M, R85A+A162C+F363I, R85A+V308M+F363M, V160S+F363M, V160S+F363I, V160S+V308M+F363I, A162L+F363M, A162C+F363M, A162C+F363I, A162C+F363L, A162C+V308M+F363M, A162C+V308L+F363M, A162L+Q179G+F363M, P306A+V308L, P306L+V308L, V308M+F363M, V308M+F363I, I343T+F363M, I343V+F363M, or N362S+F363M, in the amino acid sequence of SEQ ID NO: 5; and

a polypeptide comprising an amino acid sequence having 95% or higher homology with the amino acid sequence of said polypeptides.

【Claim 5】

A polynucleotide encoding the polypeptide of any one of claims 1 to 4.

【Claim 6】

A recombinant vector comprising the polynucleotide of claim 5.

【Claim 7】

A transformed cell comprising the recombinant vector of claim 6.

【Claim 8】

A composition for conferring or enhancing herbicide tolerance of a plant or algae, comprising one or more selected from the group consisting of the polypeptide of any one of claims 1 to 4; a polynucleotide encoding the polypeptide; a recombinant vector comprising the polynucleotide; and a transformed cell comprising the recombinant vector.

【Claim 9】

The composition of claim 8, wherein the herbicide is an herbicide inhibiting

protoporphyrinogen oxidase.

【Claim 10】

The composition of claim 9, wherein the herbicide is one or more selected from the group consisting of pyrimidinediones, diphenyl-ethers, phenylpyrazoles, N-phenylphthalimides, phenylesters, thiadiazoles, oxadiazoles, triazolinones, 5 oxazolidinediones, pyraclonil, flufenpyr-ethyl and profluazol.

【Claim 11】

The composition of claim 10, wherein the herbicide is one or more selected from the group consisting of butafenacil, saflufenacil, benzfendizone, tiafenacil, 10 fomesafen, oxyfluorfen, aclonifen, acifluorfen, bifenoxy, ethoxyfen, lactofen, chlormethoxyfen, chlorintofen, fluoroglycofen-ethyl, halosafen, pyraflufen-ethyl, fluazolate, flumioxazin, cinidon-ethyl, flumiclorac-pentyl, fluthiacet, thidiazimin, oxadiargyl, oxadiazon, carfentrazone, sulfentrazone, azafenidin, pentoxazone, pyraclonil, flufenpyr-ethyl, profluazol, phenopylate, carbamate analogues of 15 phenopylate, and agriculturally acceptable salt thereof.

【Claim 12】

The composition of claim 8, wherein the plant or algae further comprise a second herbicide-tolerant polypeptide or a gene encoding thereof, and tolerance to the second herbicide is conferred or enhanced.

20 **【Claim 13】**

The composition of claim 12, wherein the second herbicide is selected from the group consisting of glyphosate, glufosinate, dicamba, 2,4-D(2,4-Dichlorophenoxyacetic acid), isoxaflutole, ALS(acetolactate synthase)-inhibiting herbicide, photosystem II-inhibiting herbicide, phenylurea-based herbicide, bromoxynil-based herbicide, and 25 combinations thereof.

【Claim 14】

The composition of claim 12, wherein the second herbicide-tolerant polypeptide is one or more selected from the group consisting of:

glyphosate herbicide-tolerant EPSPS (glyphosate resistant 5-enolpyruvylshikimate-3-phosphate synthase), GOX (glyphosate oxidase), GAT 30

(glyphosate-N-acetyltransferase) or glyphosate decarboxylase;

glufosinate herbicide-tolerant PAT (phosphinothricin-N-acetyltransferase);

dicamba herbicide-tolerant DMO (dicamba monooxygenase);

2,4-D (2,4-dichlorophenoxyacetic acid) herbicide-tolerant 2,4-D

5 monooxygenase or AAD (aryloxyalkanoate dioxygenase);

ALS (acetolactate synthase)-inhibiting sulfonylurea-based herbicide-tolerant

AHAS (acetohydroxyacid synthase), AHAS (acetohydroxyacid synthase) or atahasl (acetohydroxyacid synthase large subunit);

photosystem II-inhibiting herbicide-tolerant photosystem II protein D1;

10 phenylurea herbicide-tolerant Cytochrome P450;

plastid-inhibiting herbicide-tolerant HPPD (hydroxylphenylpyruvate dioxygenase);

bromoxynil herbicide-tolerant nitrilase; and

combinations thereof.

15 **【Claim 15】**

The composition of claim 12, wherein the gene encoding the second herbicide-tolerant polypeptide is one or more selected from the group consisting of:

glyphosate herbicide-tolerant cp4 epsps, mepsps, 2mepsps, goxv247, gat4601 or gat4621 gene;

20 glufosinate herbicide-tolerant BAR or PAT gene;

dicamba herbicide-tolerant dmo gene;

2,4-D(2,4-dichlorophenoxyacetic acid) herbicide-tolerant AAD-1 or AAD-12 gene;

isoxaflutole herbicide-tolerant HPPDPF W336 gene;

25 sulfonylurea herbicide-tolerant ALS, Csr1, Csr1-1, Csr1-2, GM-HRA, S4-HRA, Zm-HRA, SurA or SurB gene;

photosystem II-inhibiting herbicide-tolerant psbA gene;

phenylurea herbicide-tolerant CYP76B1 gene;

bromoxynil herbicide-tolerant bxn gene; and

30 combinations thereof.

【Claim 16】

A transformant of a plant or algae having herbicide tolerance, or a clone or progeny thereof, comprising the polypeptide of any one of claims 1 to 4 or a polynucleotide encoding thereof.

5 **【Claim 17】**

The transformant, clone, or progeny thereof of claim 16, wherein the transformant is a plant cell, protoplast, callus, hypocotyl, seed, cotyledon, shoot, or whole plant.

【Claim 18】

10 A method of preparing a transgenic plant or algae having herbicide tolerance, the method comprising transforming algae, a plant cell, protoplast, callus, hypocotyl, seed, cotyledon, shoot, or whole plant with the polypeptide of any one of claims 1 to 4 or a polynucleotide encoding thereof.

【Claim 19】

15 A method of conferring or enhancing herbicide tolerance of a plant or algae, the method comprising transforming algae, a plant cell, protoplast, callus, hypocotyl, seed, cotyledon, shoot, or whole plant with the polypeptide of any one of claims 1 to 4 or a polynucleotide encoding thereof.

【Claim 20】

20 A method of controlling weeds in a cropland, the method comprising:
providing the cropland with a plant comprising the polypeptide of any one of claims 1 to 4 or a polynucleotide encoding thereof, and
applying an effective dosage of protoporphyrinogen oxidase-inhibiting herbicide to the cropland.

25 **【Claims 21】**

The method of claim 20, wherein the step of applying an effective dosage of protoporphyrinogen oxidase-inhibiting herbicide to the cropland is performed by applying an effective dosage of two or more kinds of protoporphyrinogen oxidase-inhibiting herbicides sequentially or simultaneously.

30 **【Claim 22】**

The method of claim 20,

wherein the plant further comprises a second herbicide-tolerant polypeptide or a gene encoding thereof, and

the step of applying an effective dosage of protoporphyrinogen oxidase-inhibiting herbicide to the cropland is performed by applying an effective dosage of the
5 protoporphyrinogen oxidase-inhibiting herbicide and a second herbicide are applied sequentially or simultaneously.

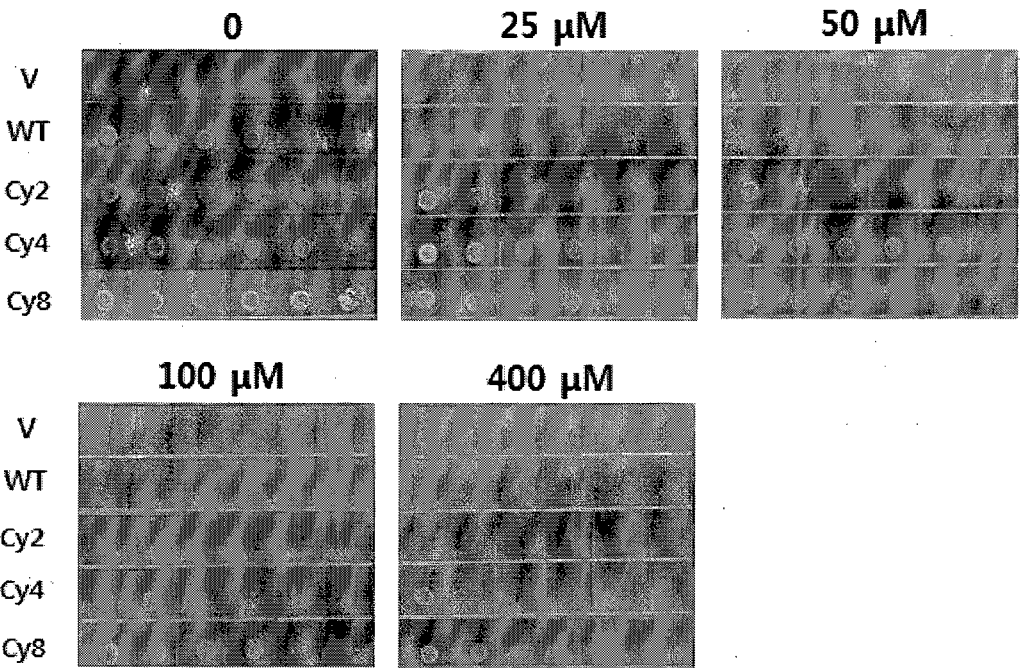
【Claim 23】

A method of removing an undesired aquatic organism from a culture media, the
10 method comprising:

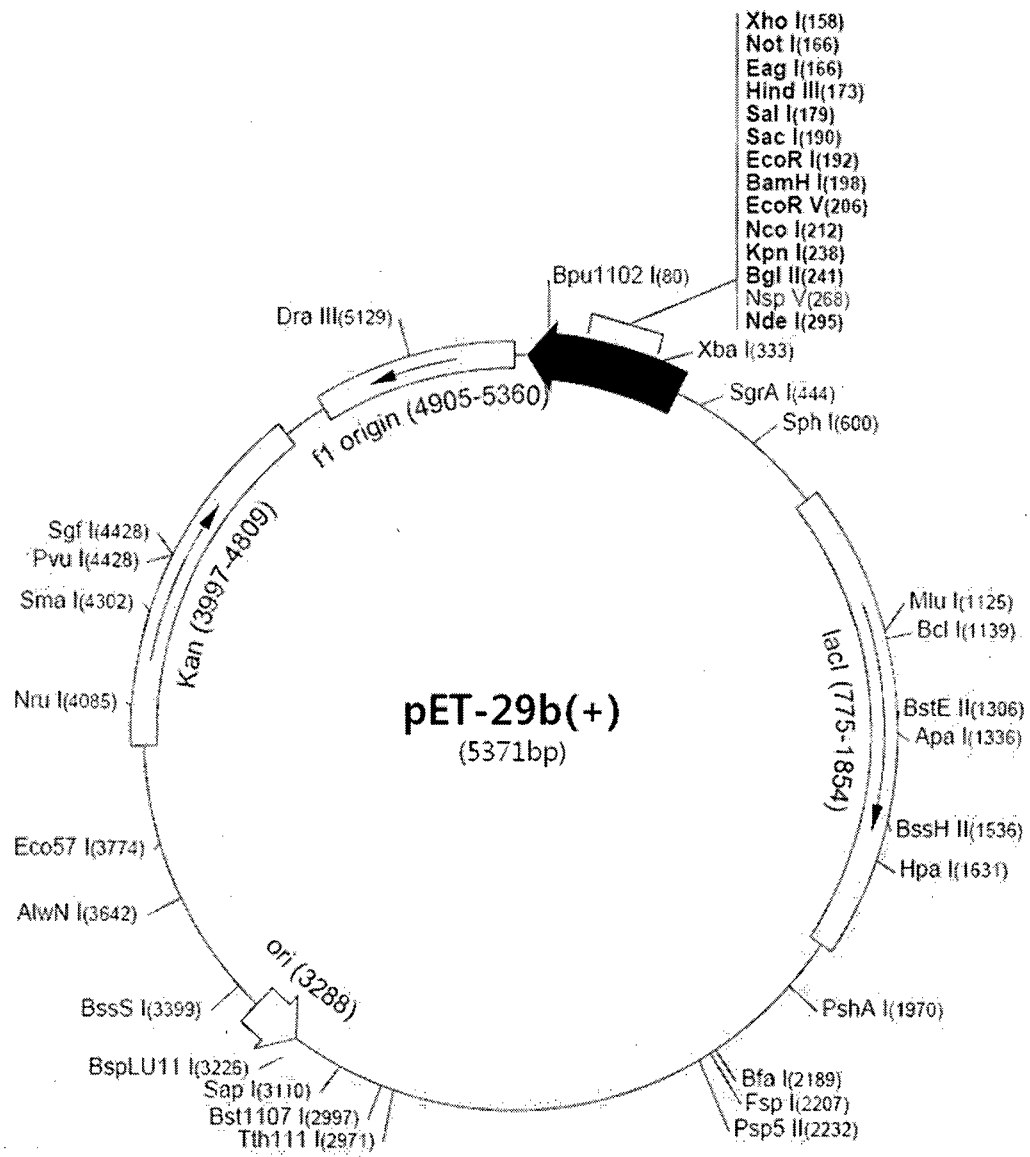
providing a culture media with algae comprising the polypeptide of any one of claims 1 to 4 or a polynucleotide encoding thereof, and

applying an effective dosage of protoporphyrinogen oxidase-inhibiting herbicide to the culture media.

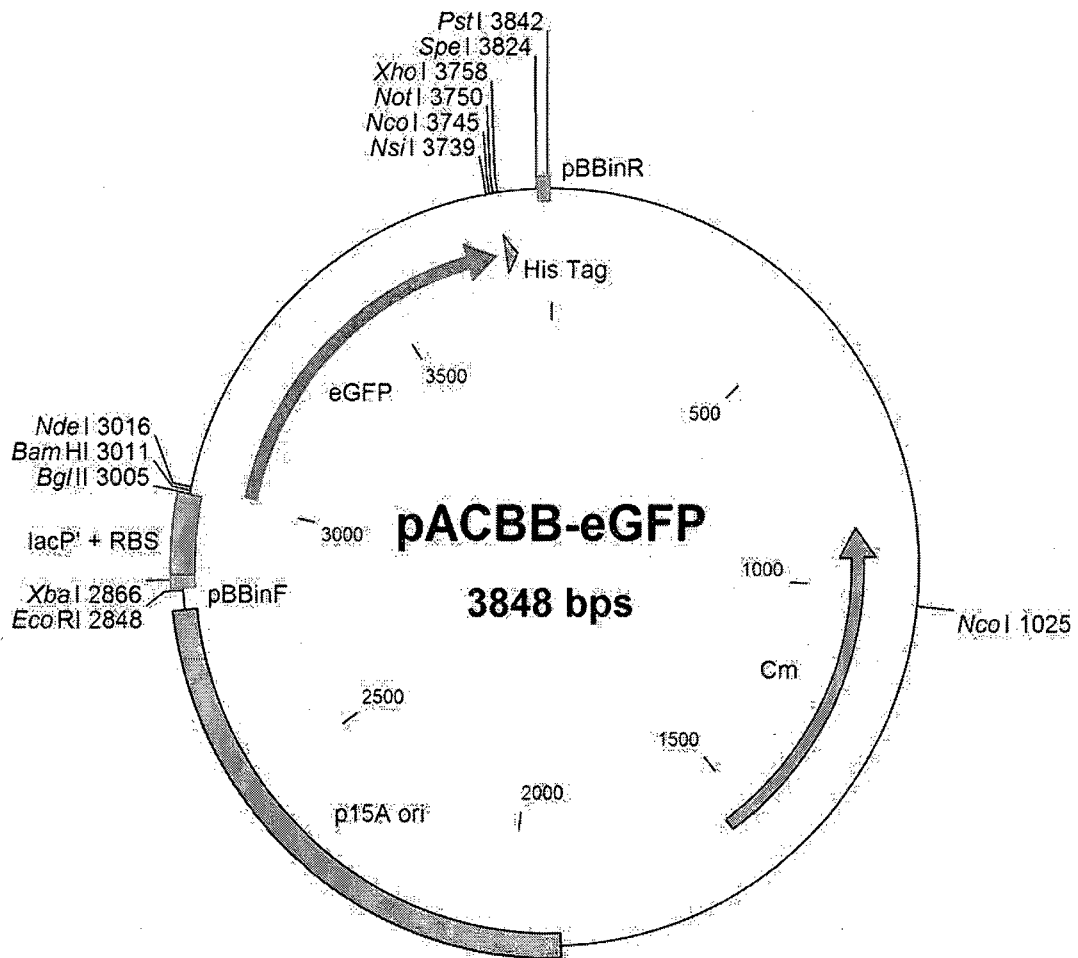
【Fig. 1】



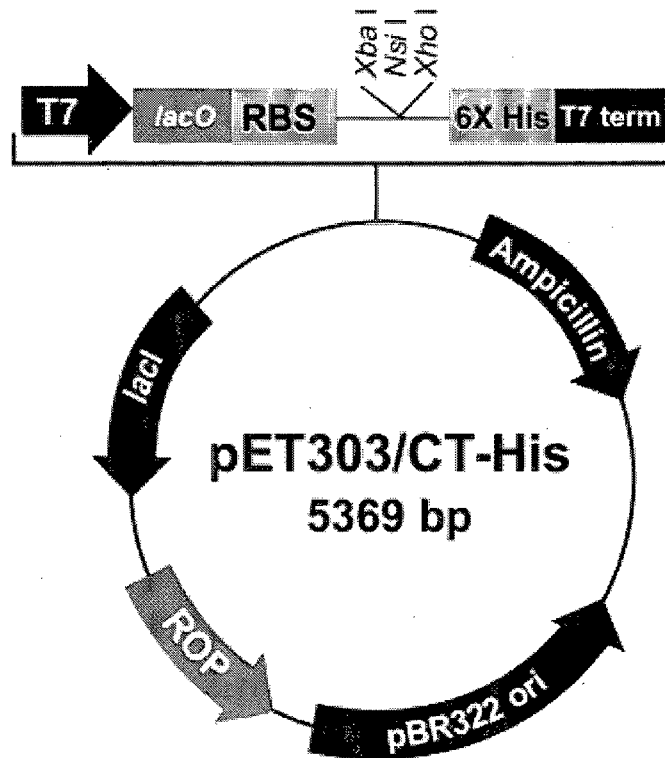
【Fig. 2】



【Fig. 3】



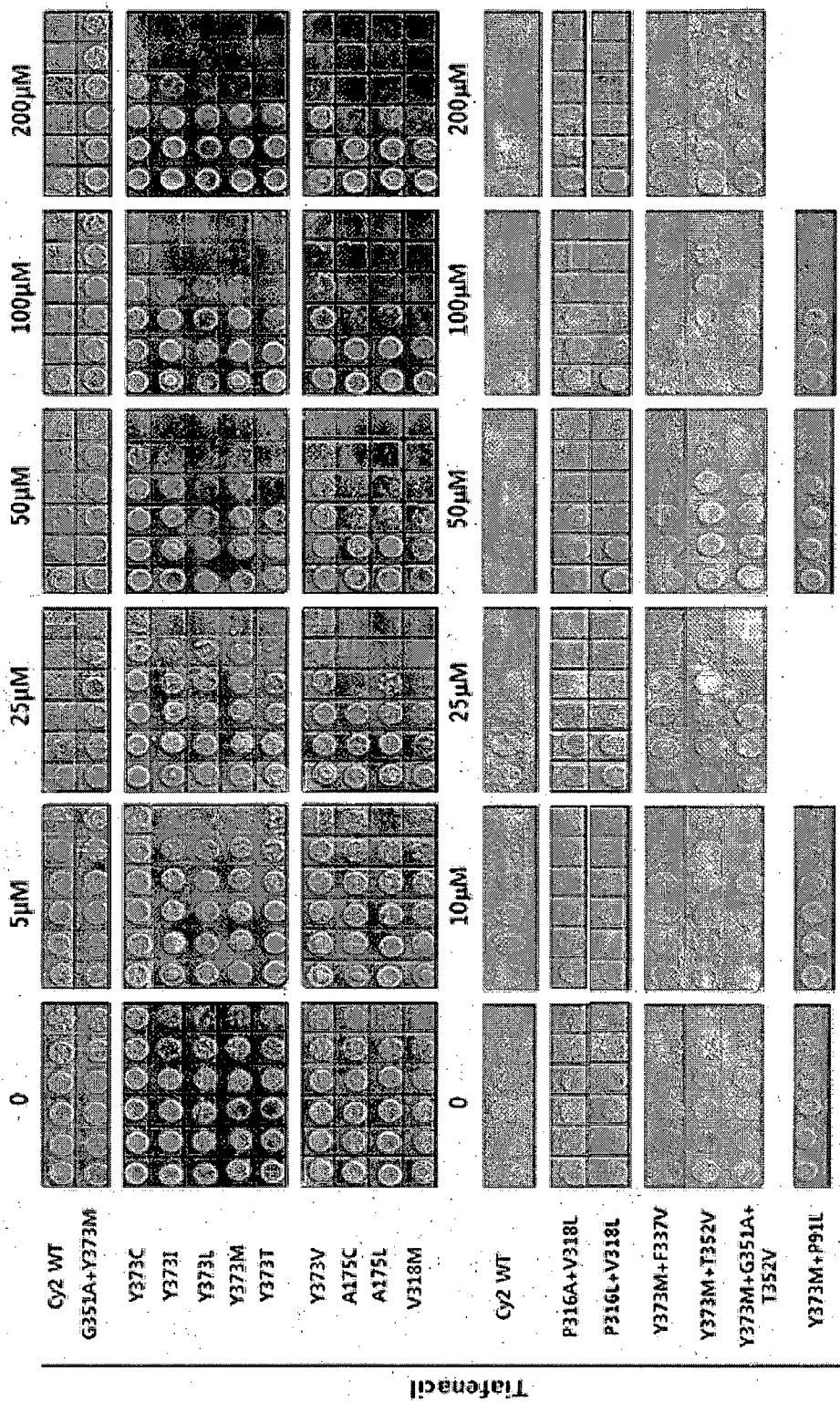
【Fig. 4】



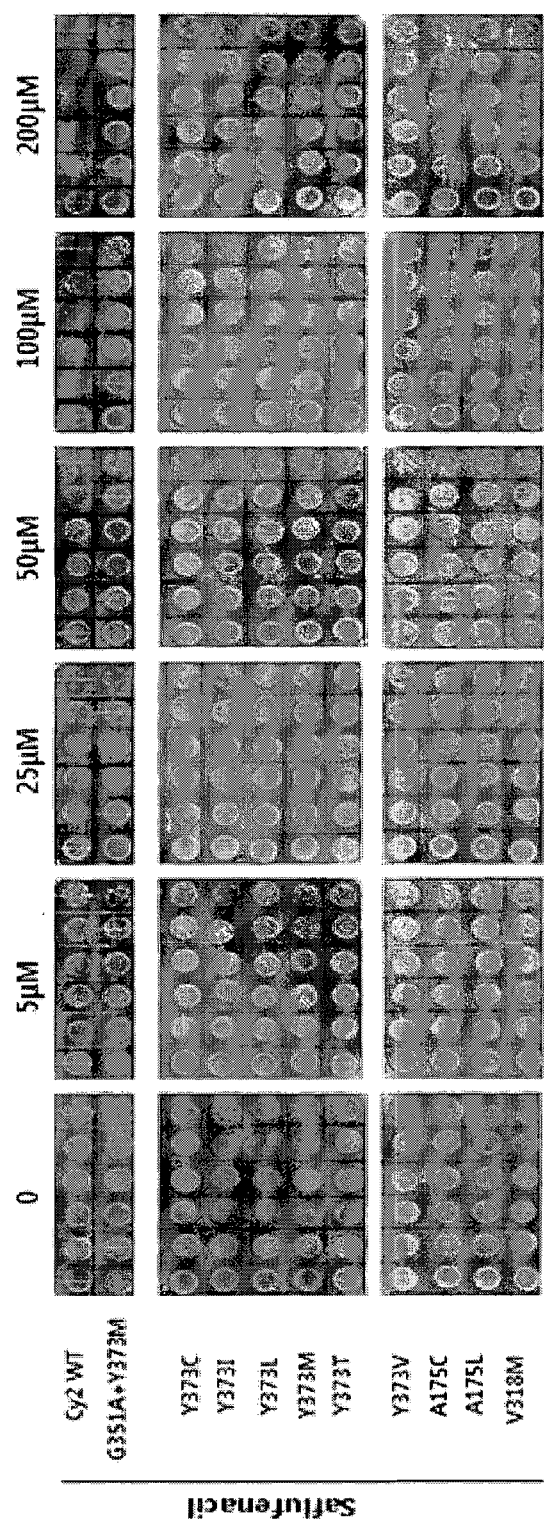
**Comments for pET303 CT-His
5369 nucleotides**

T7 promoter: bases 20-36
 T7 promoter priming site: bases 20-39
 lac operator (*lacO*): bases 39-63
 Ribosome binding site (RBS): bases 95-100
 6X His Tag: bases 119-136
 T7 reverse priming site: bases 186-206
 T7 transcription termination region: bases 147-277
 F1 origin: bases 287-742
bla promoter: bases 775-879
 Ampicillin (*bla*) resistance gene: bases 874-1734
 pBR322 origin: bases 1945-2678 (c)
ROP ORF: bases 2920-3011 (c)
lacI ORF: bases 3914-5032 (c)

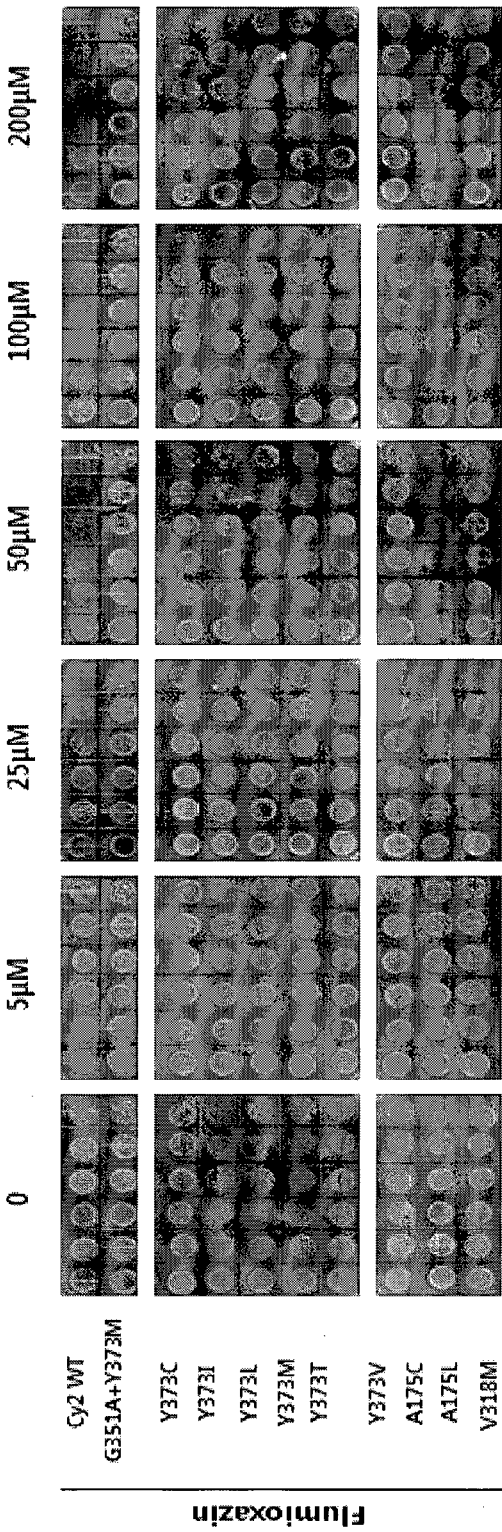
【Fig. 5】



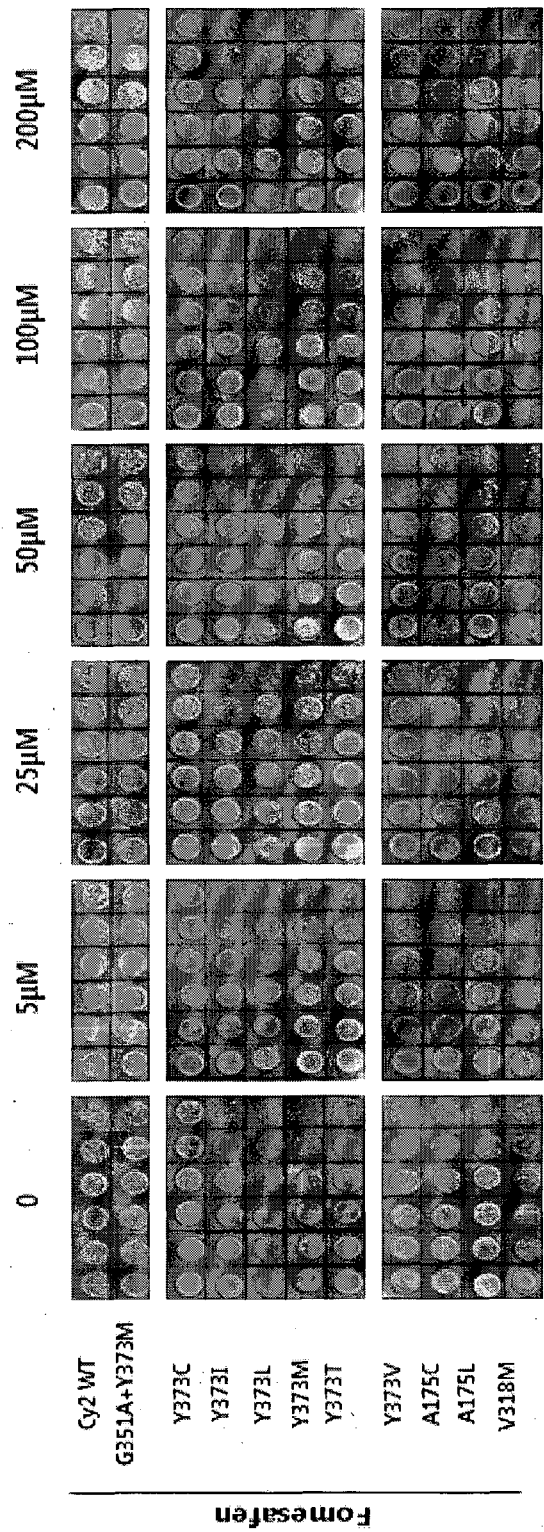
【Fig. 6】



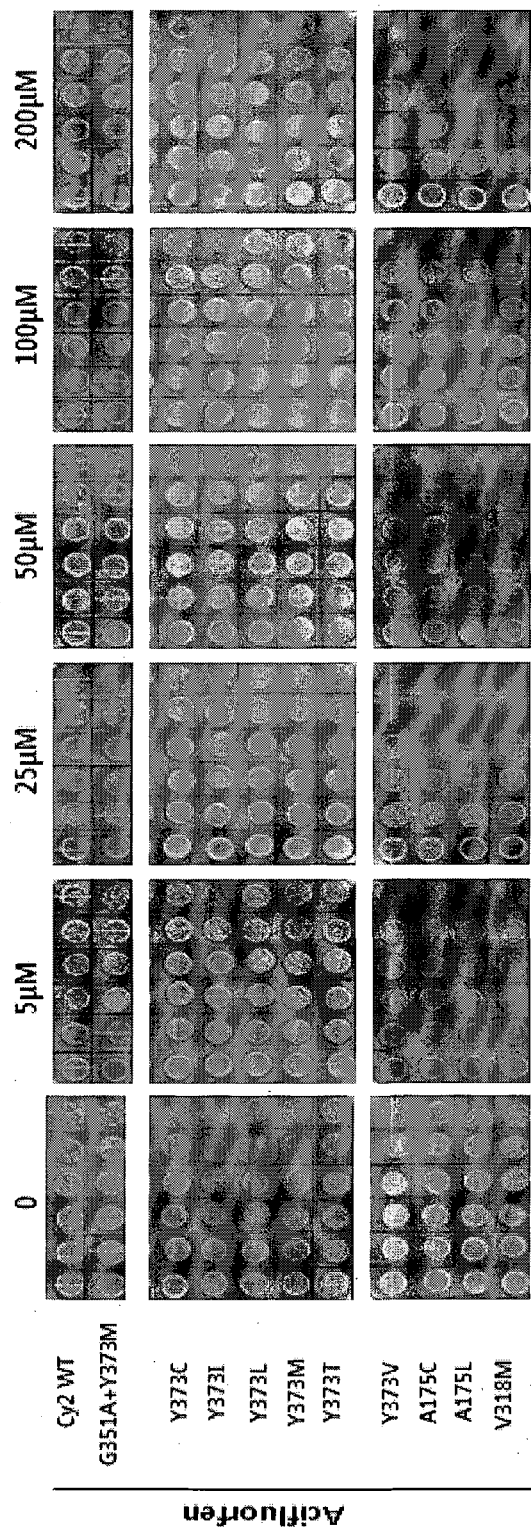
【Fig. 7】



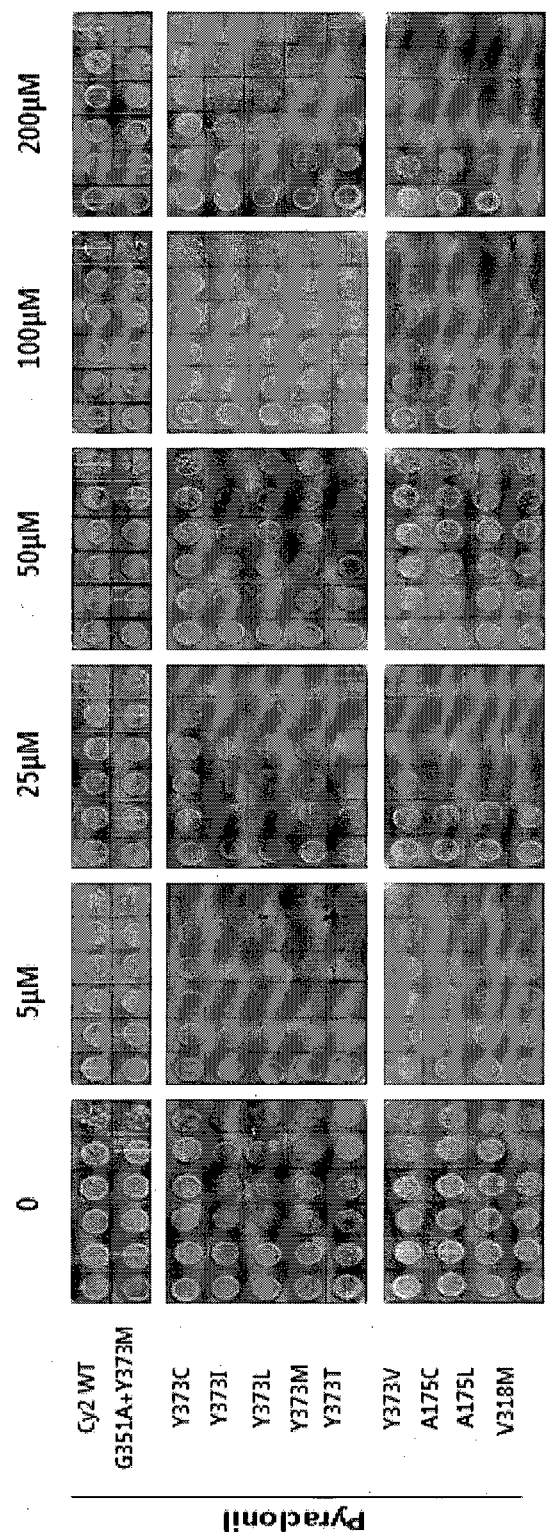
【Fig. 8】



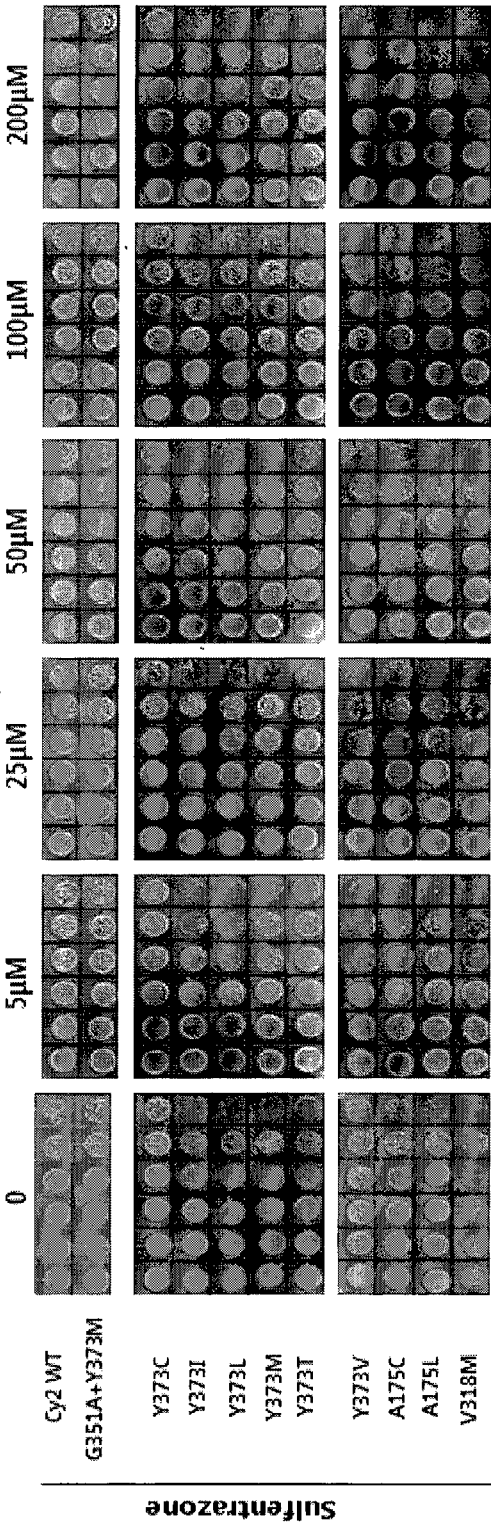
【Fig. 9】



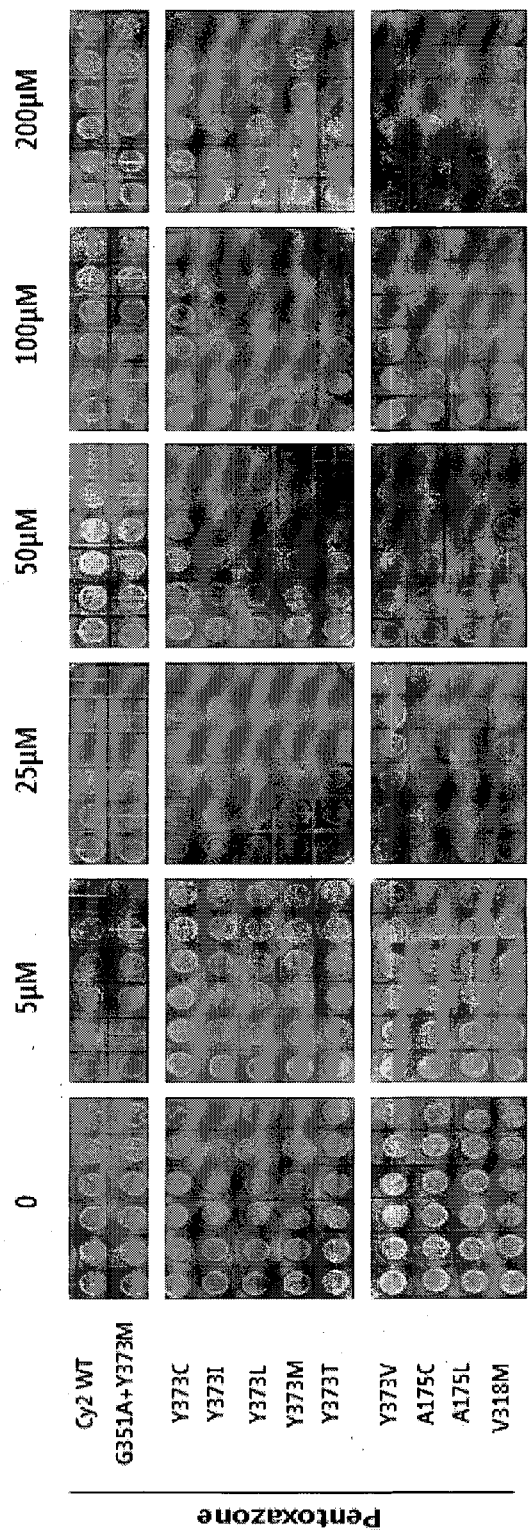
【Fig. 10】



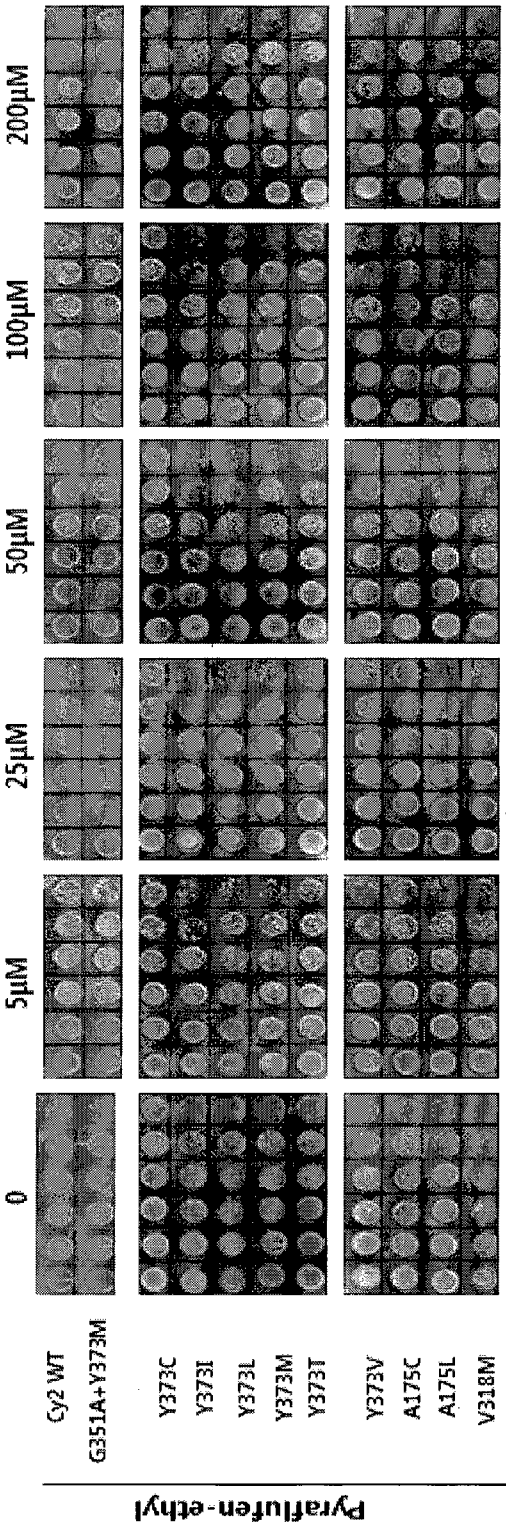
【Fig. 11】



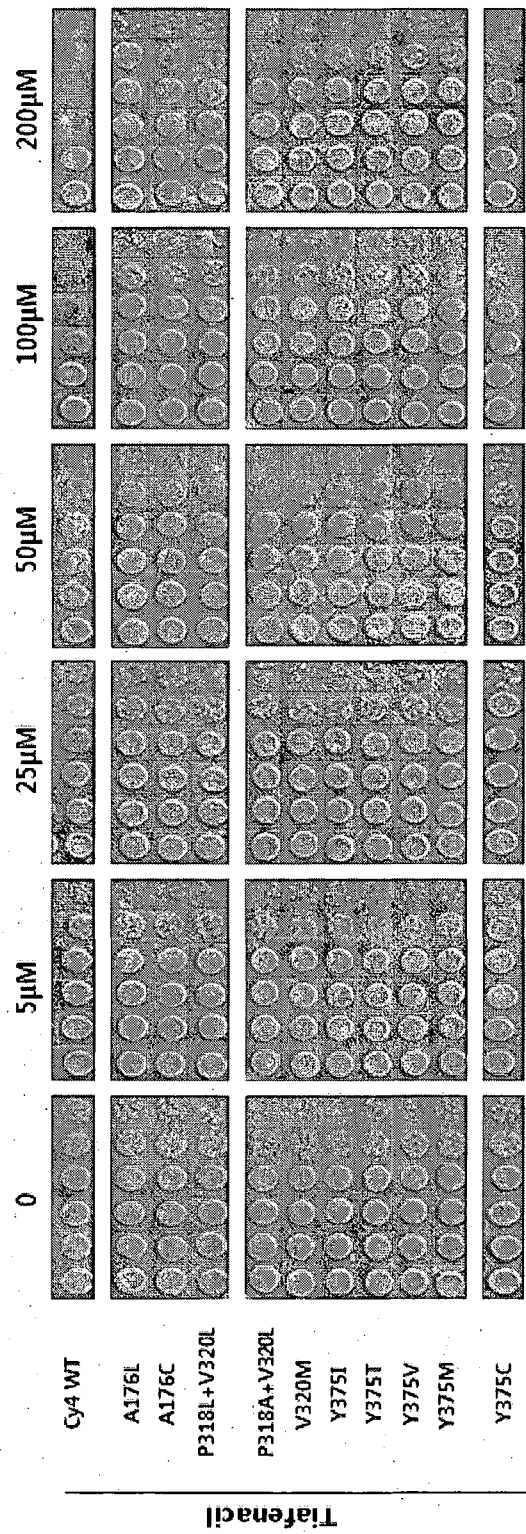
【Fig. 12】



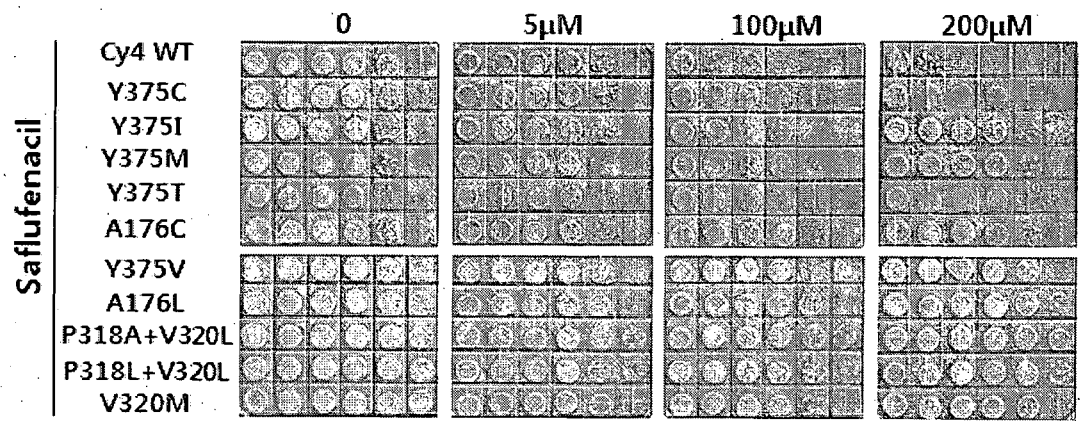
【Fig. 13】



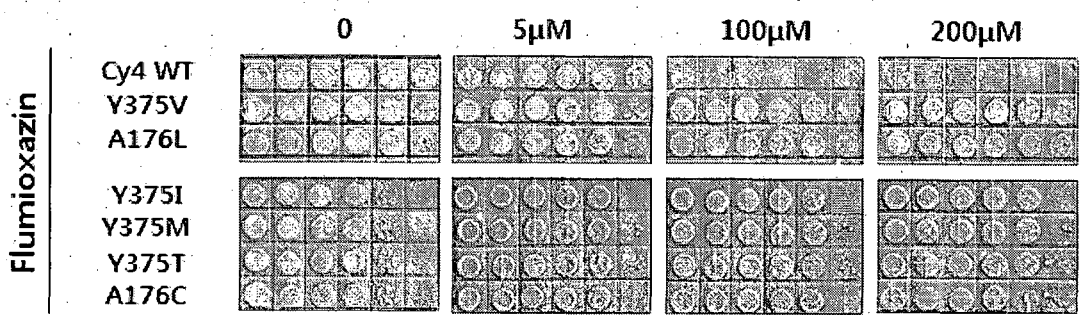
【Fig. 14】



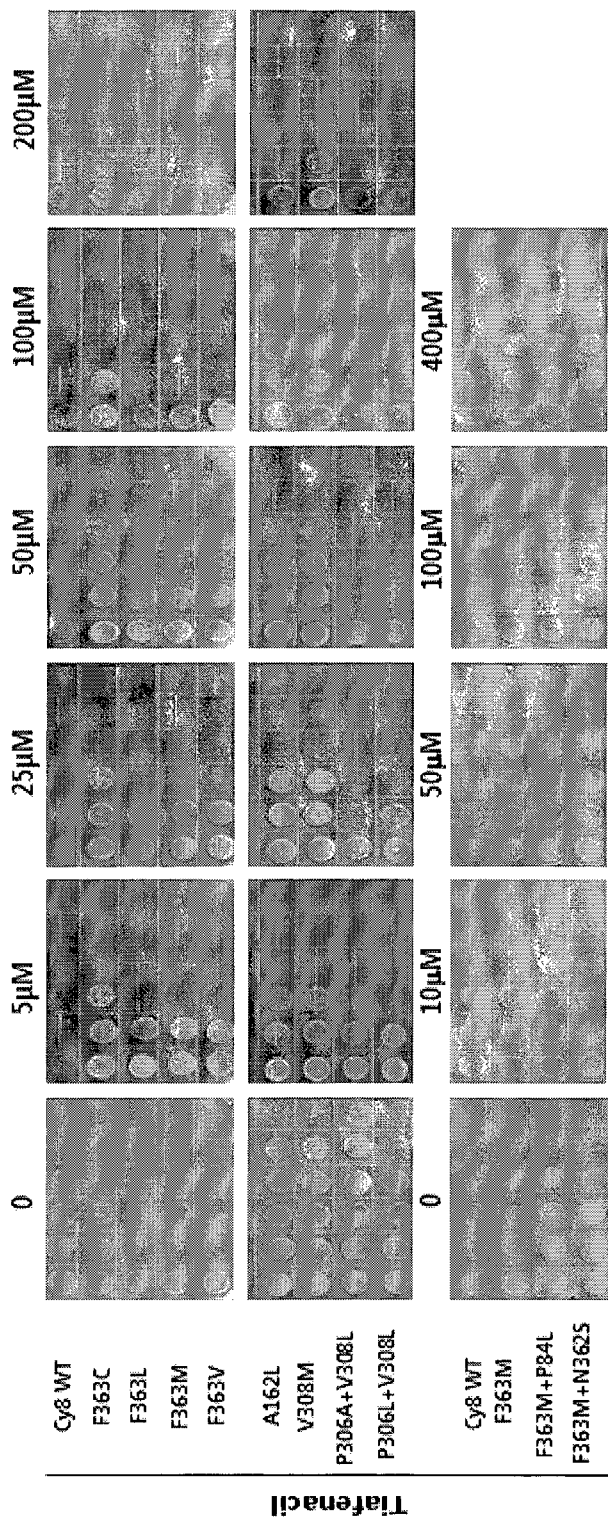
【Fig. 15】



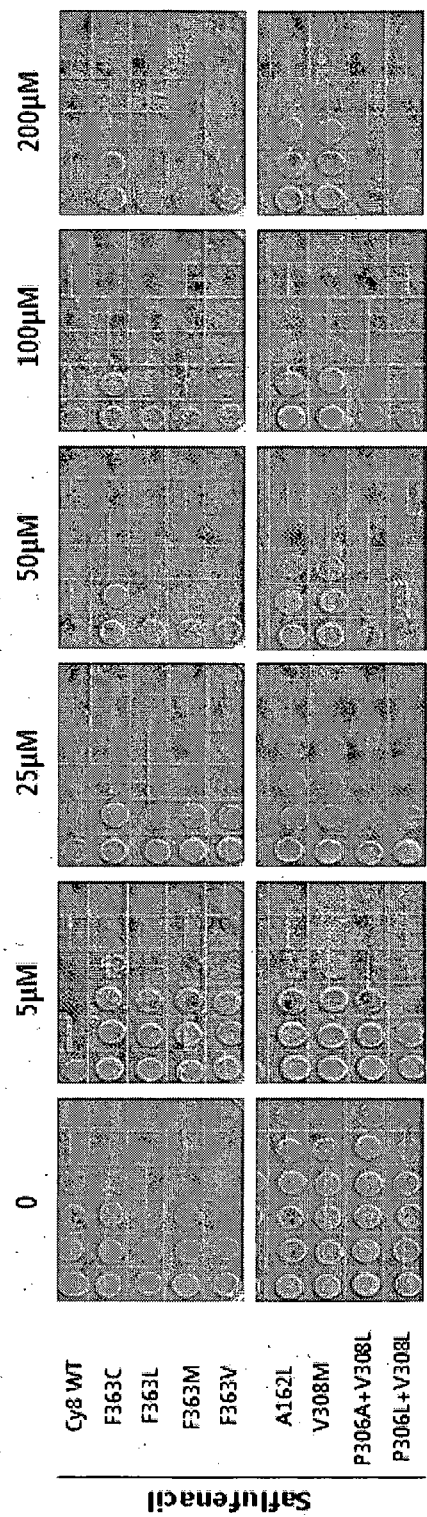
【Fig. 16】



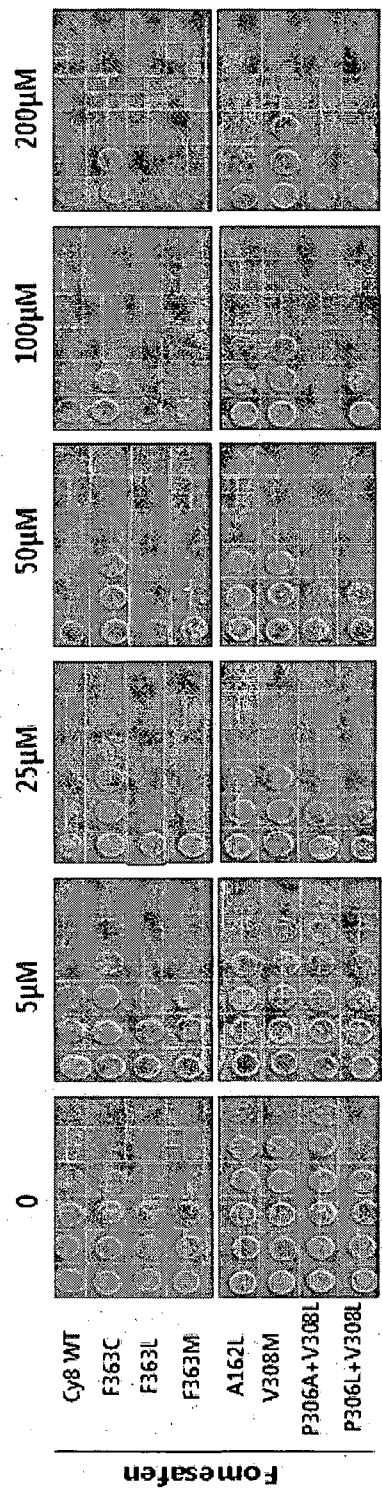
【Fig. 17】



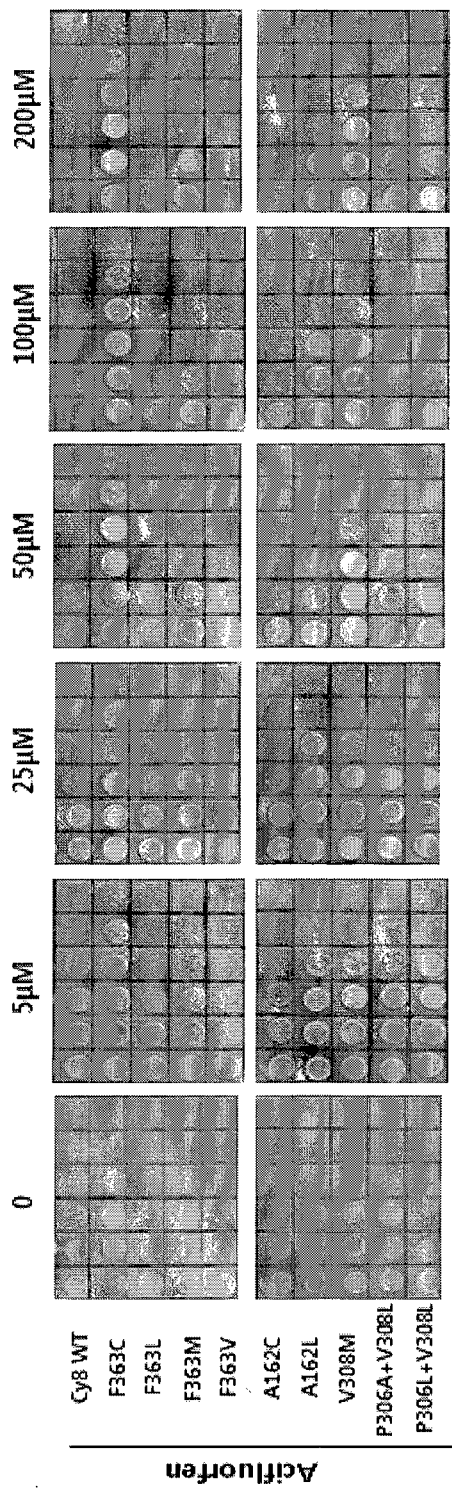
【Fig. 18】



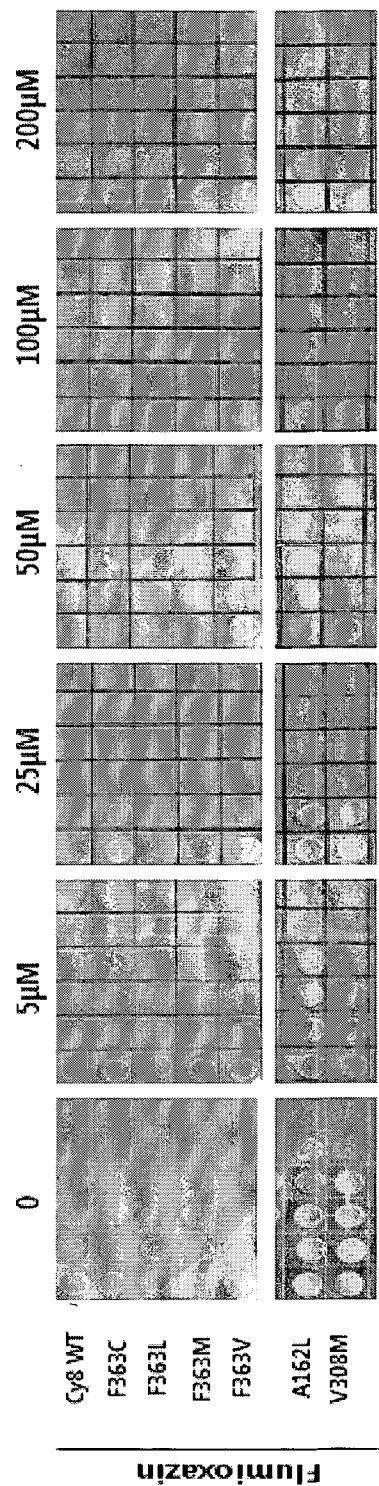
【Fig. 19】



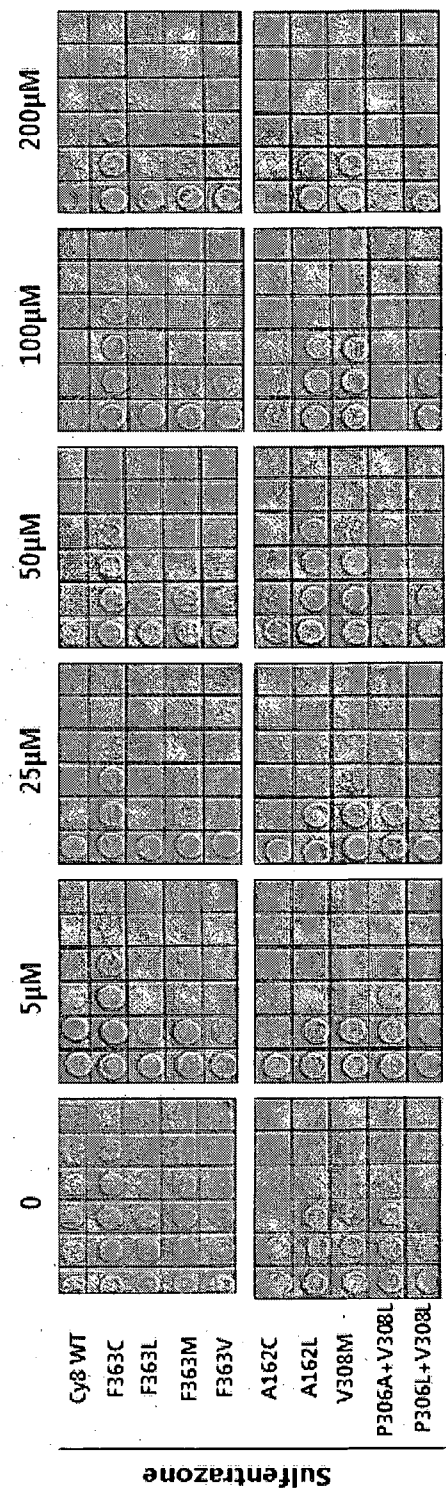
【Fig. 20】



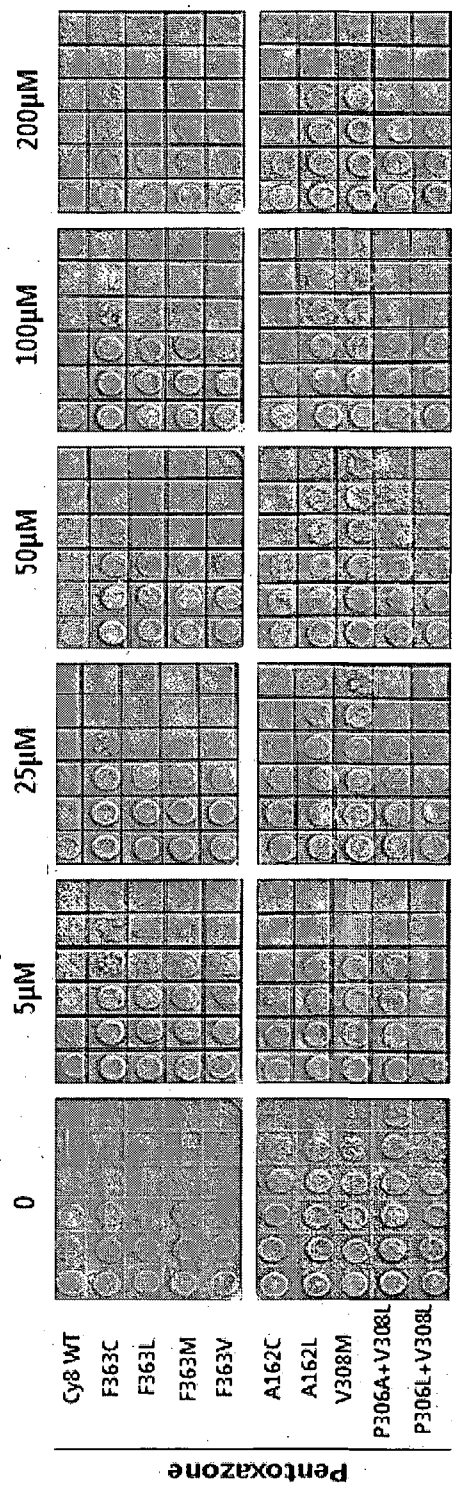
【Fig. 21】



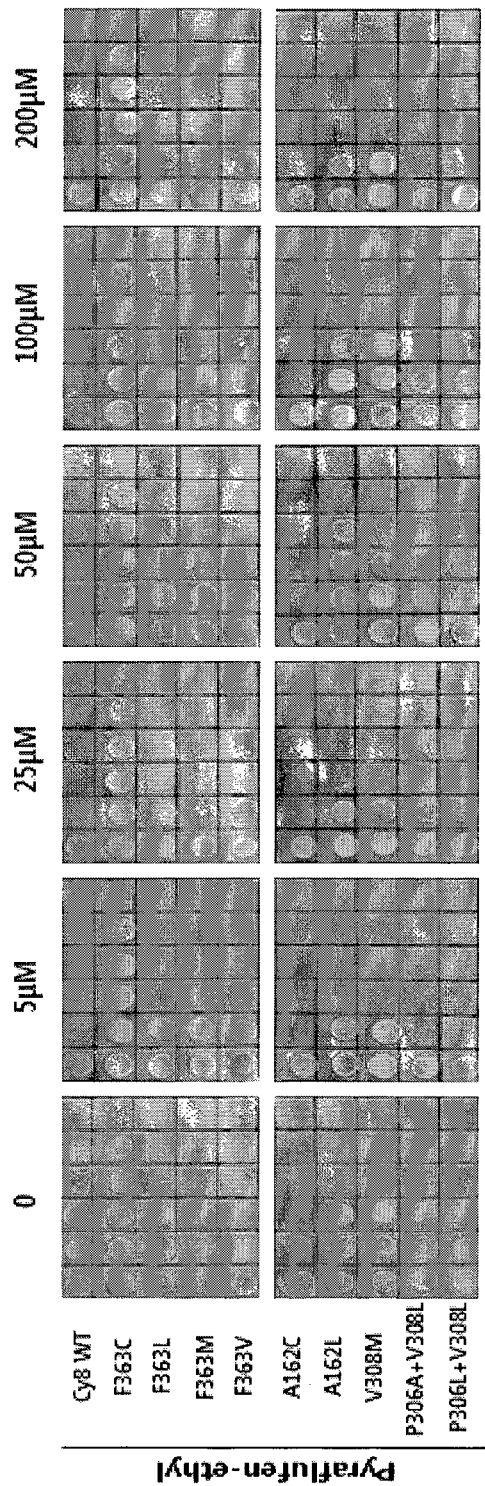
【Fig. 22】



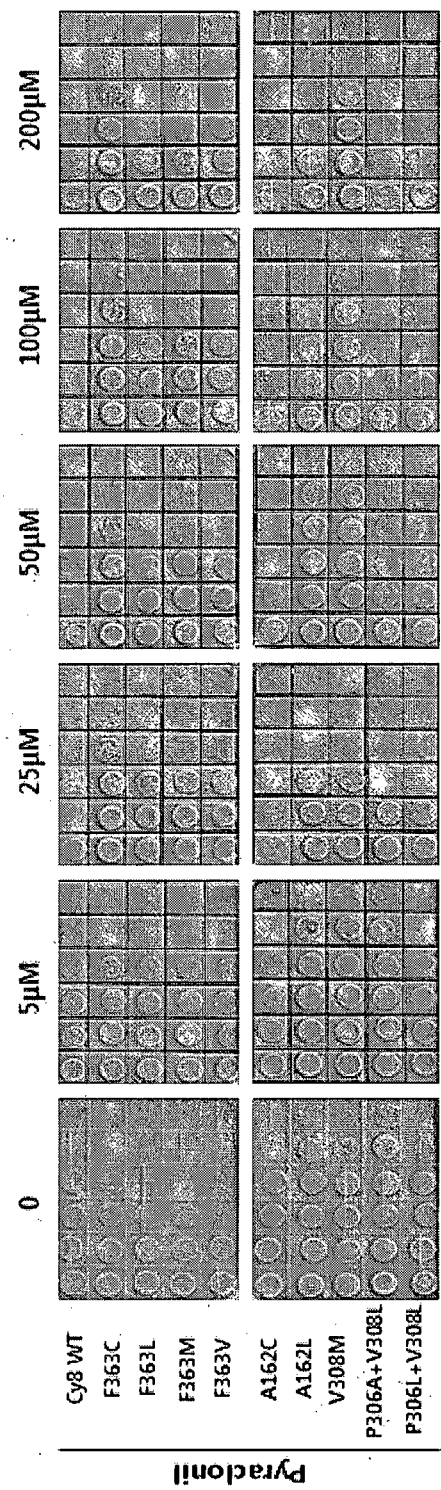
【Fig. 23】



【Fig. 24】

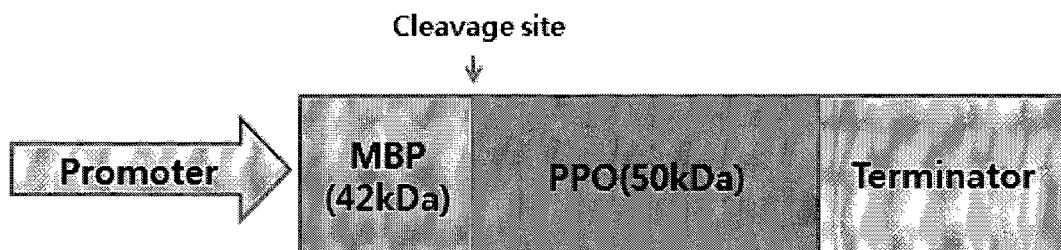


【Fig. 25】

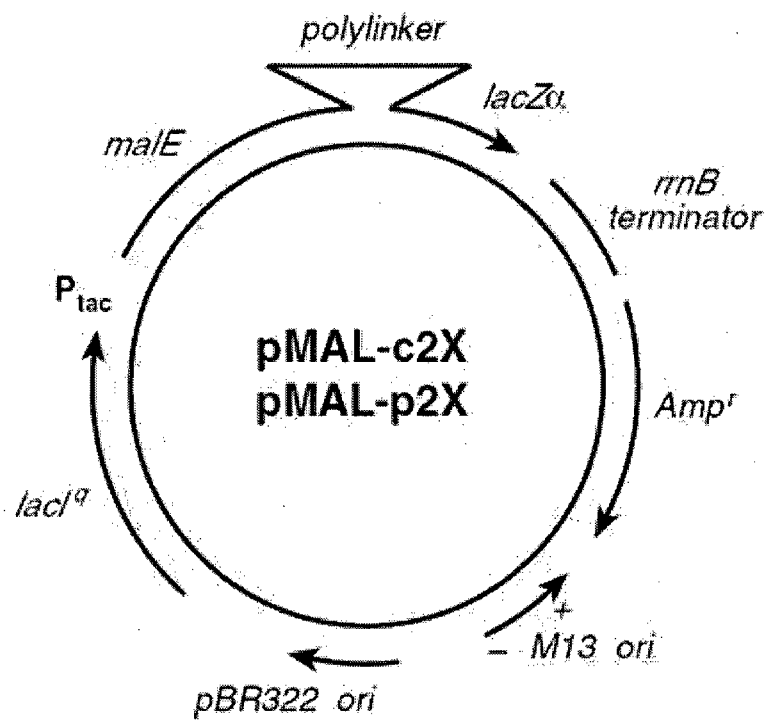


【Fig. 26】

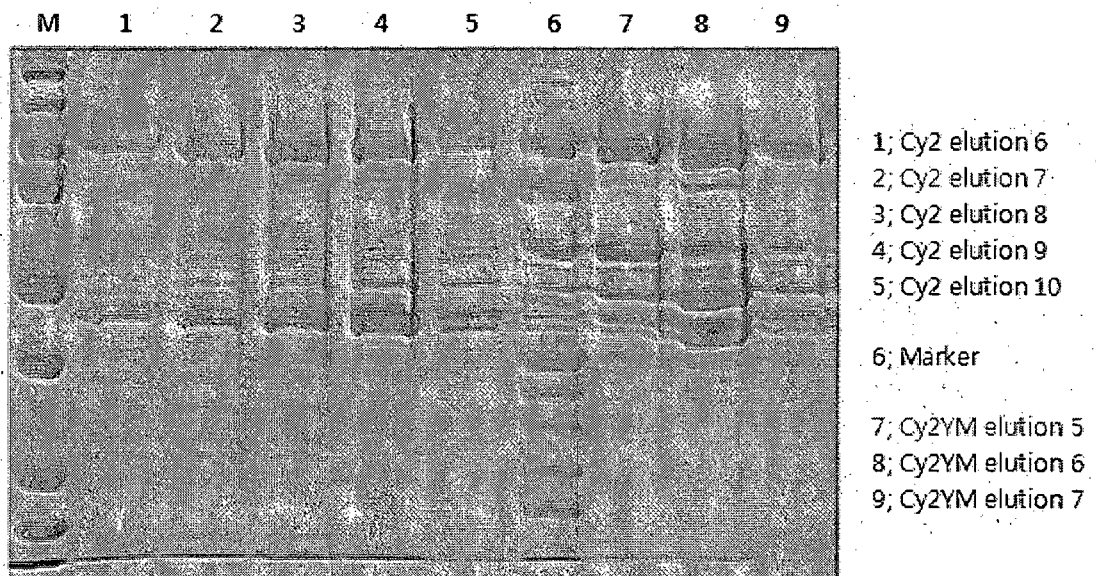
Vector for Fusion Protein of PPO and MBP(Maltose binding protein)



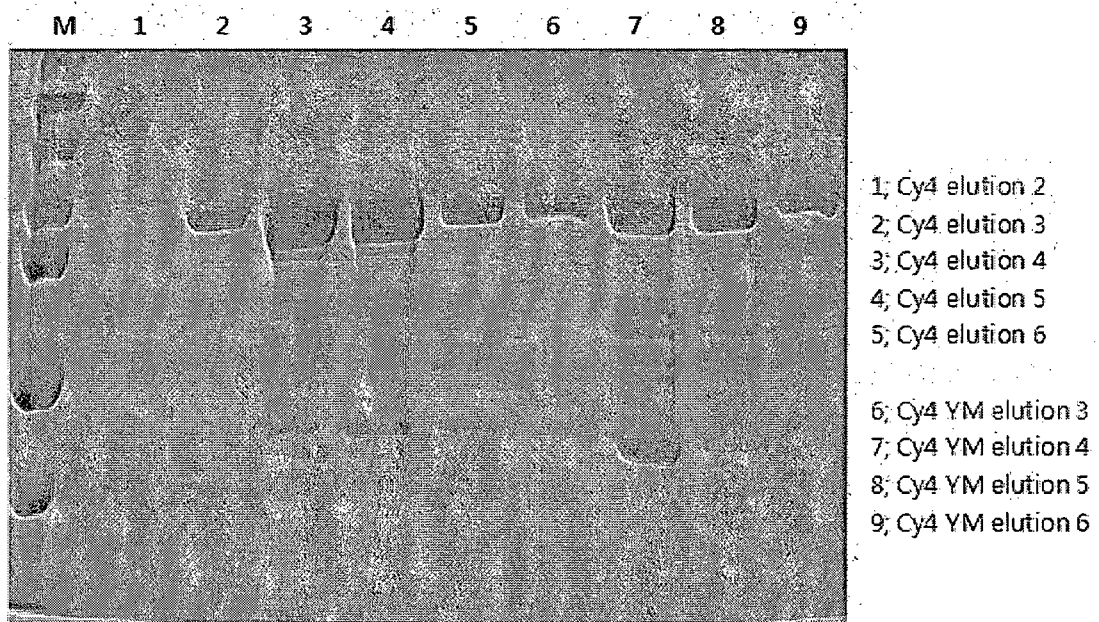
【Fig. 27】



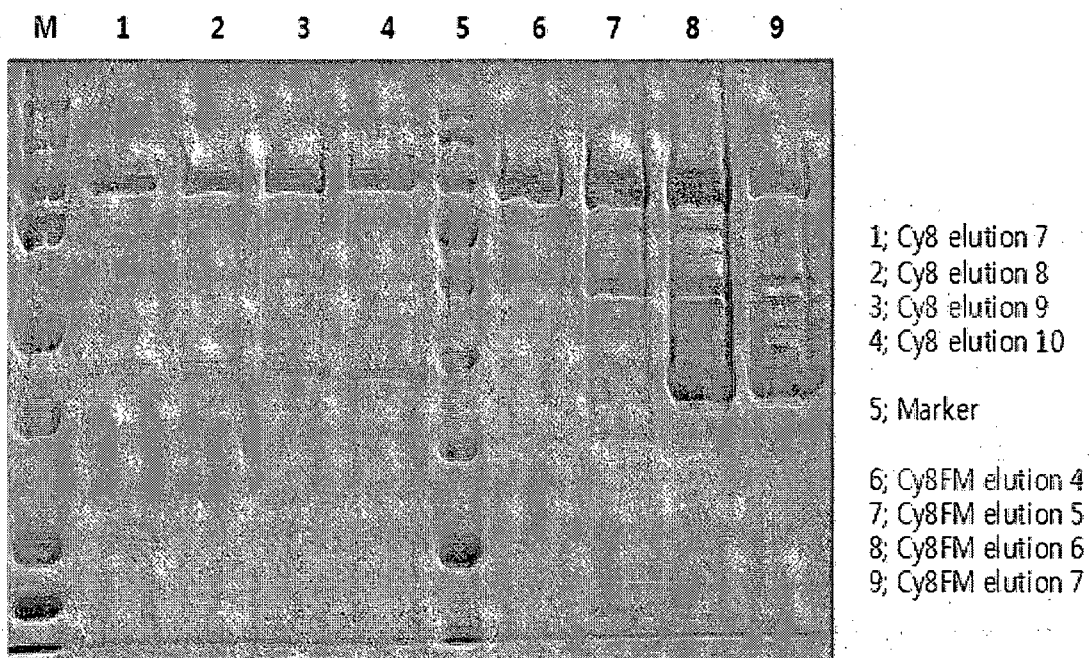
【Fig. 28】



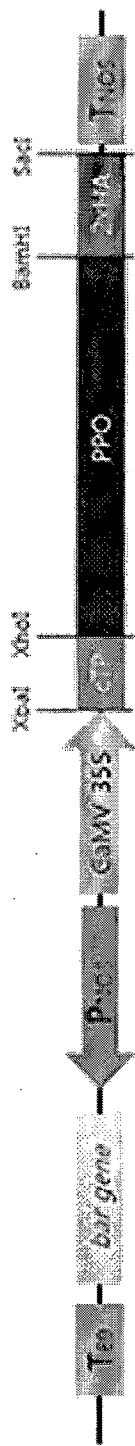
【Fig. 29】



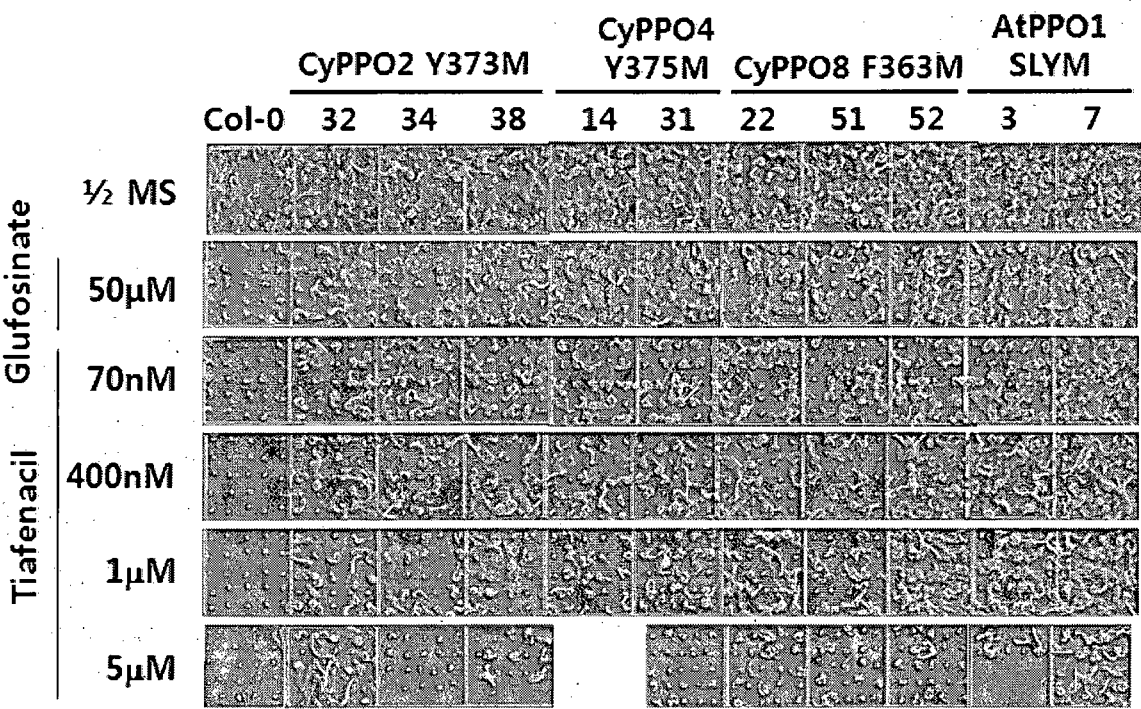
【Fig. 30】



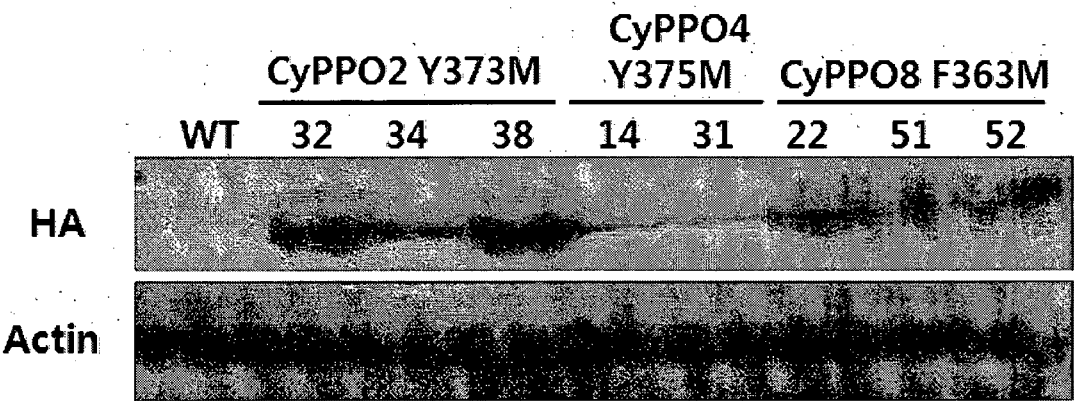
【Fig. 31】



【Fig. 32】

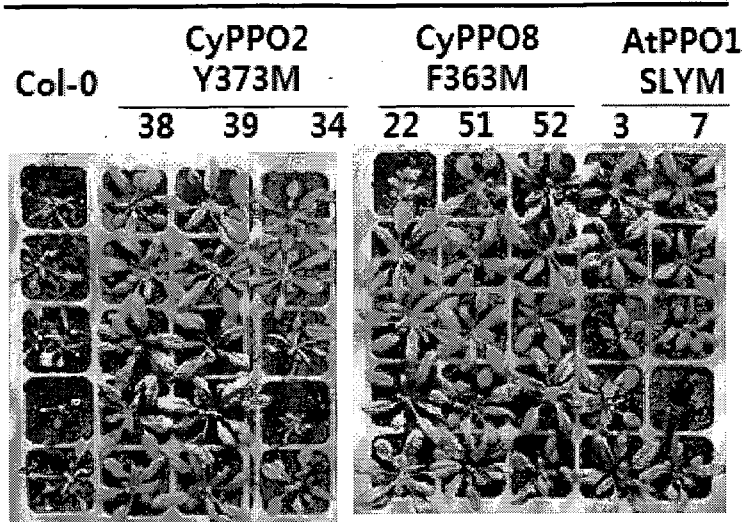


【Fig. 33】



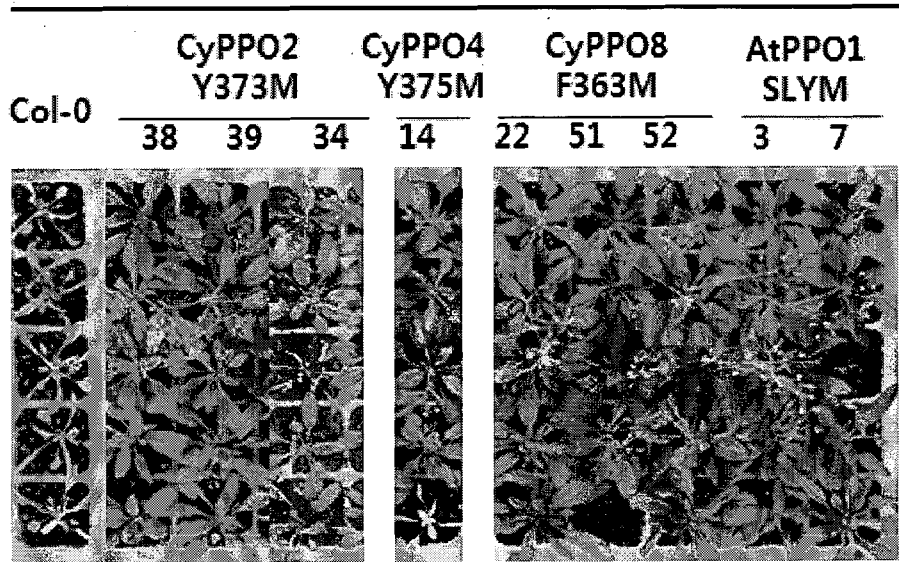
【Fig. 34】

Tiafenacil



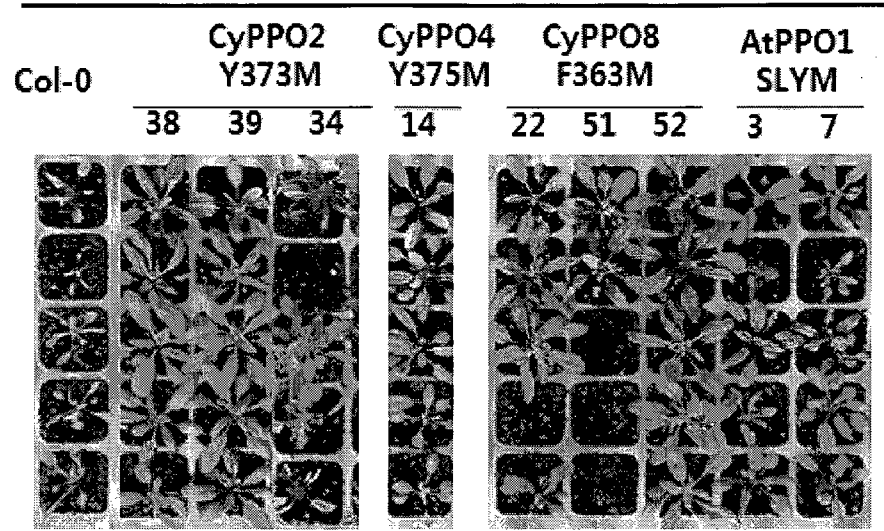
【Fig. 35】

Saflufenacil

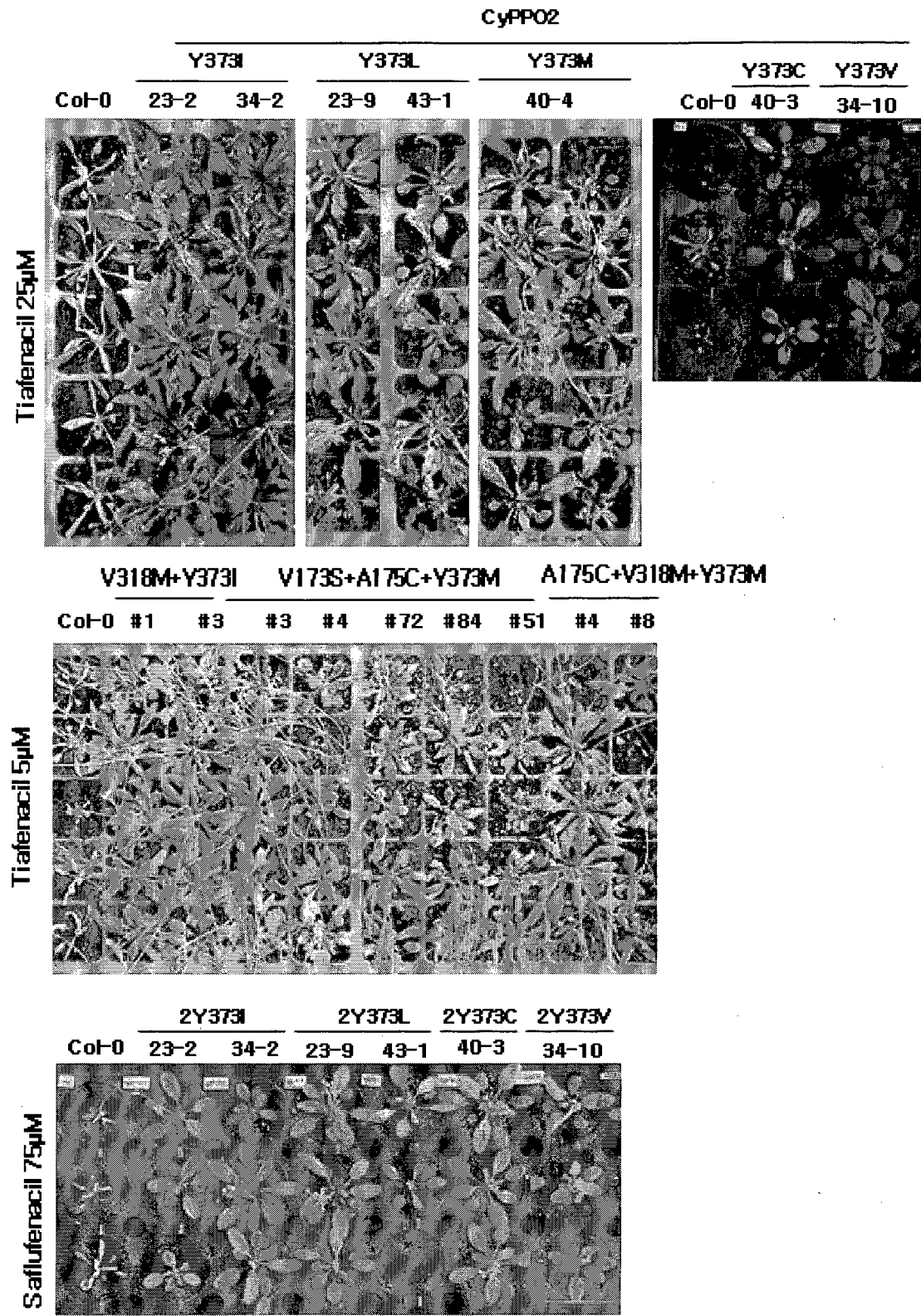


【Fig. 36】

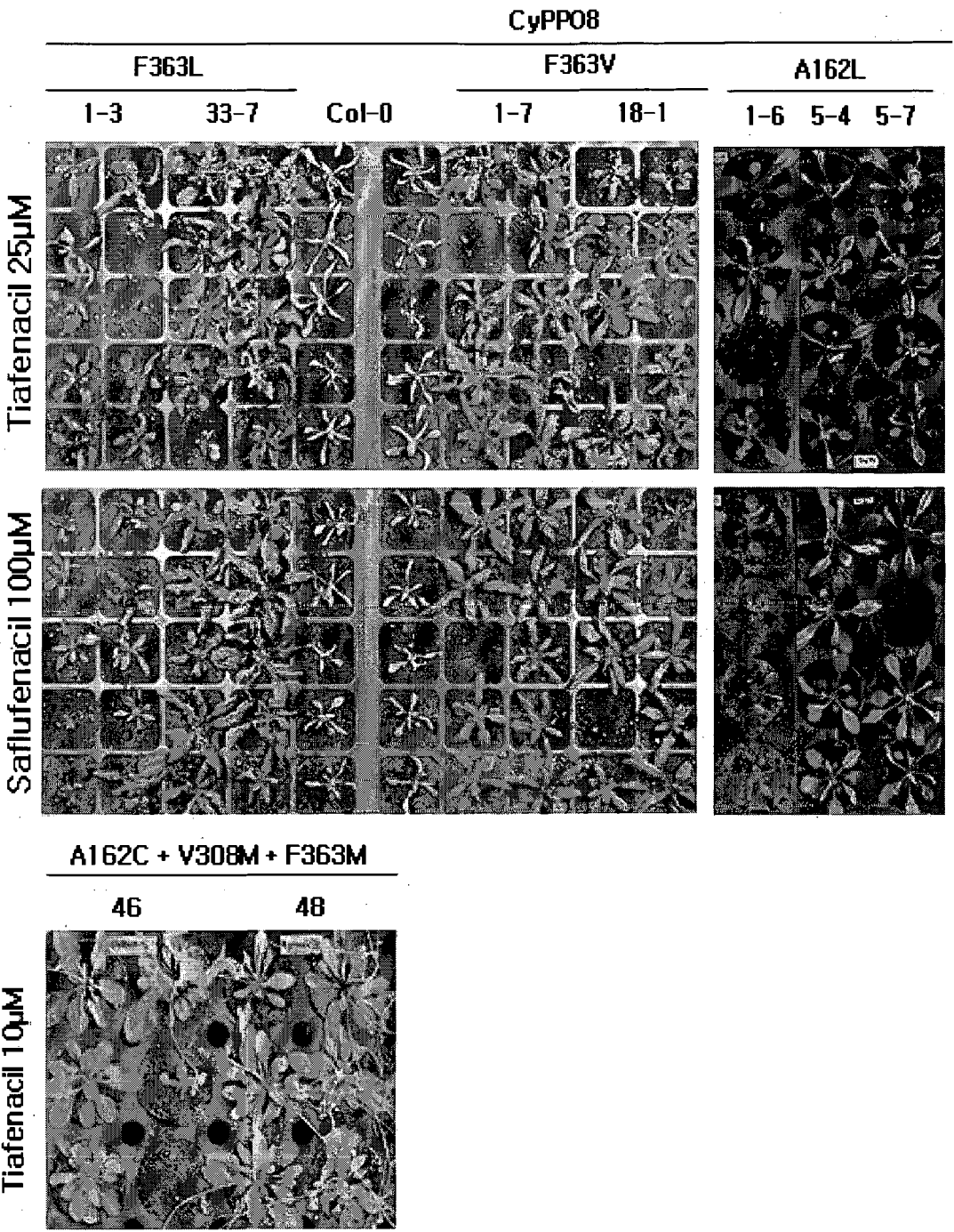
Fomesafen



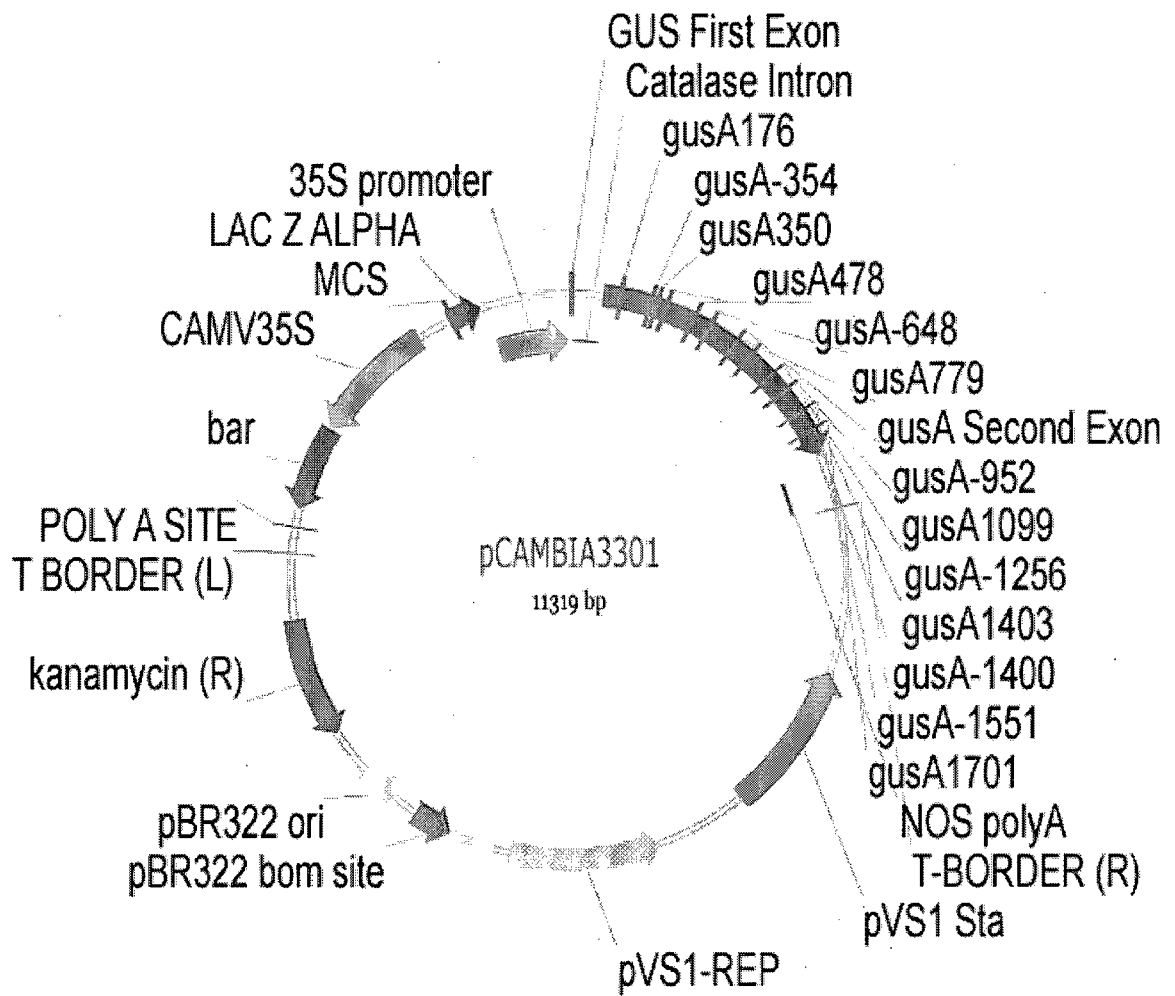
【Fig. 37】



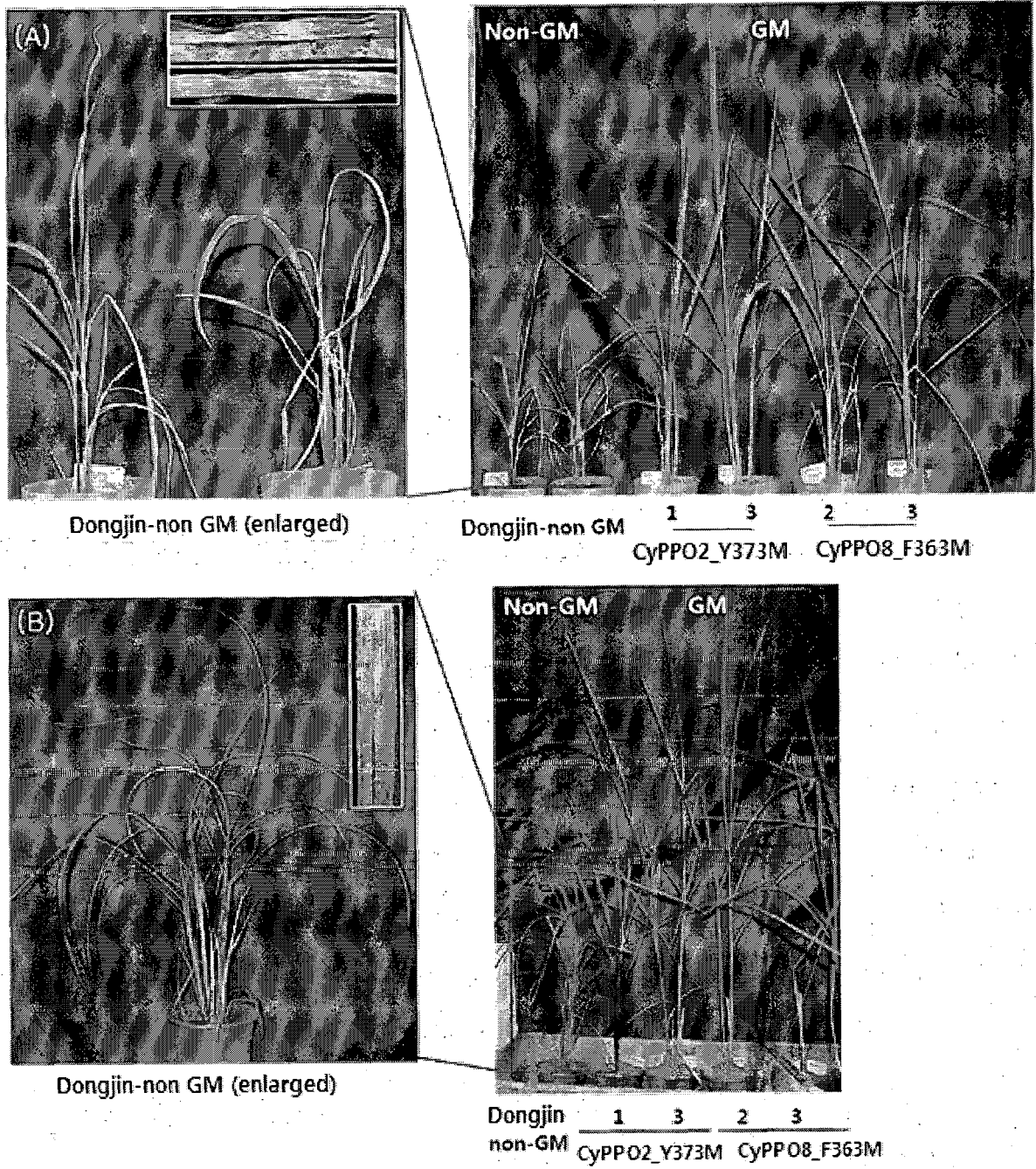
【Fig. 38】

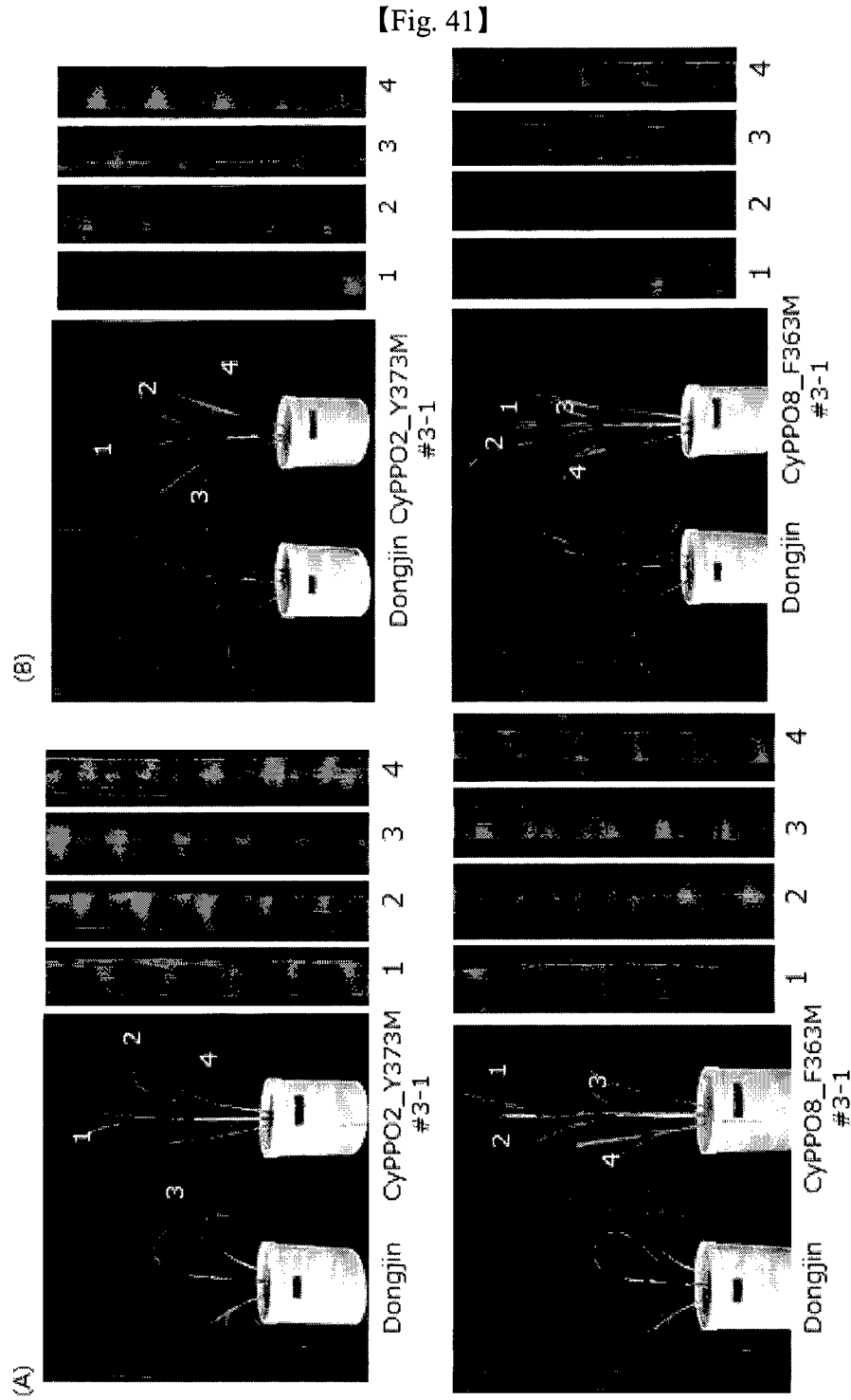


【Fig. 39】

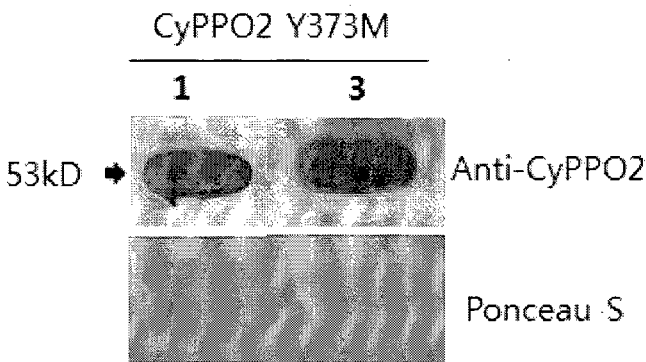


【Fig. 40】

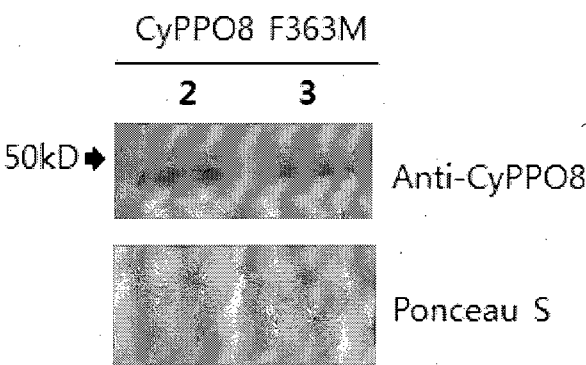




【Fig. 42】

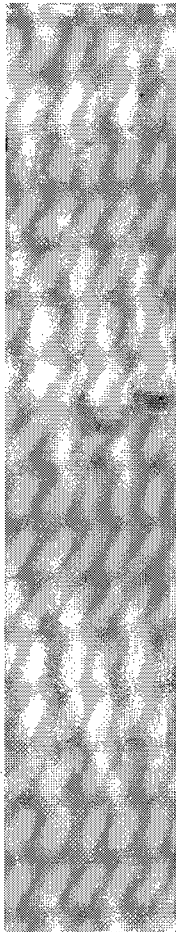


【Fig. 43】

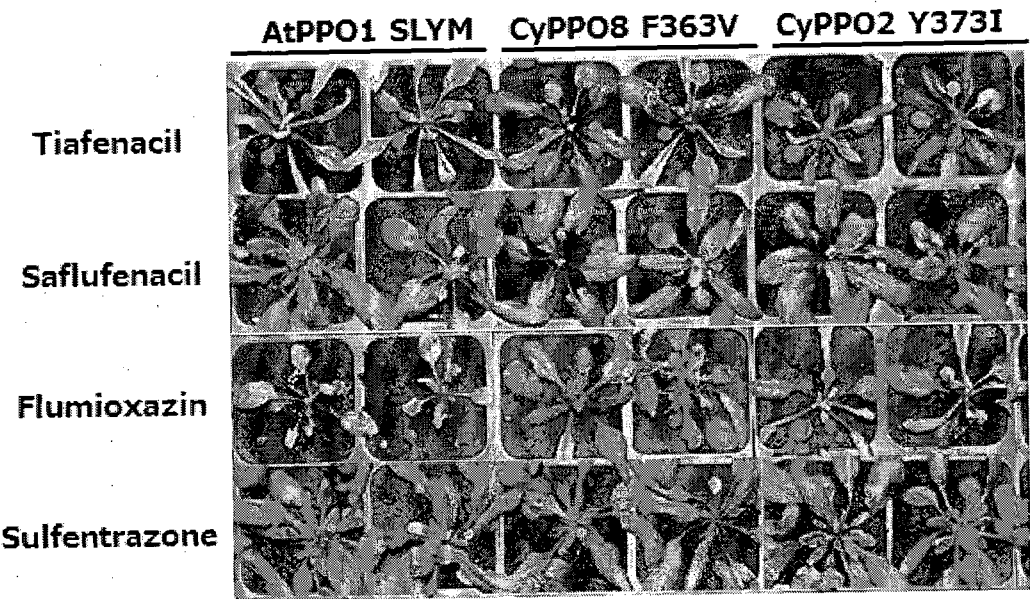


【Fig. 44】

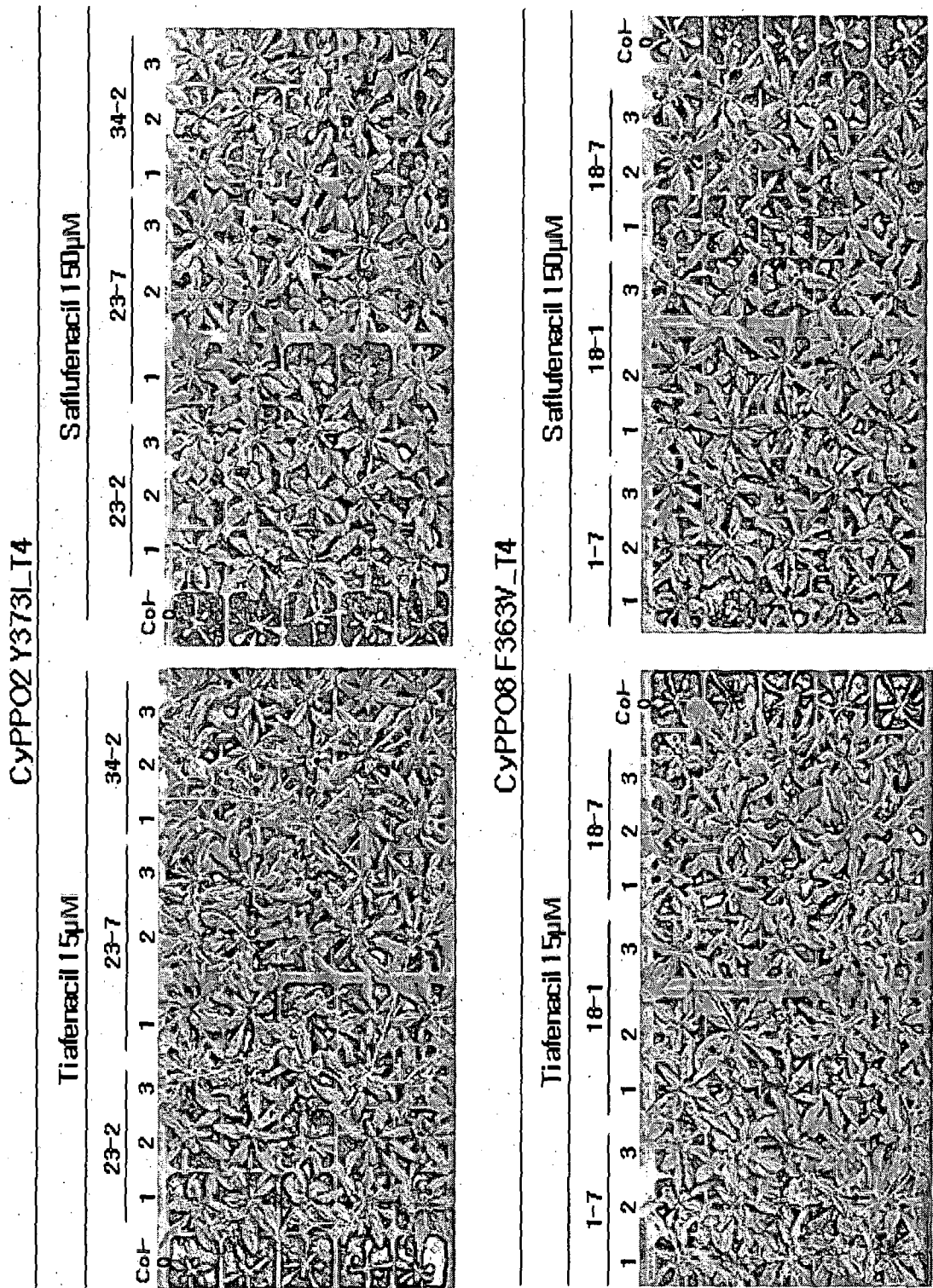
CyPPO8 F363M
Wild type #3 #6



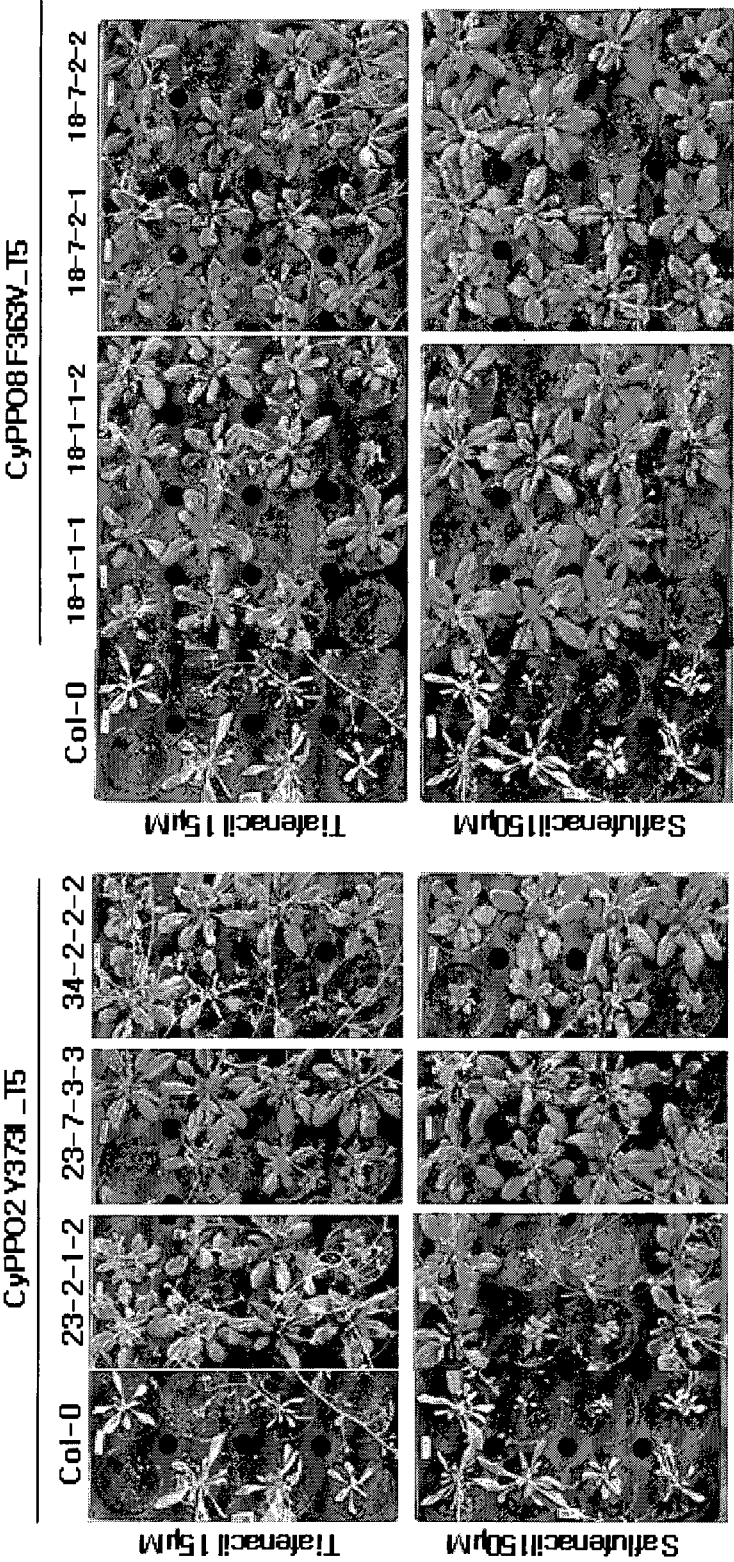
【Fig. 45】



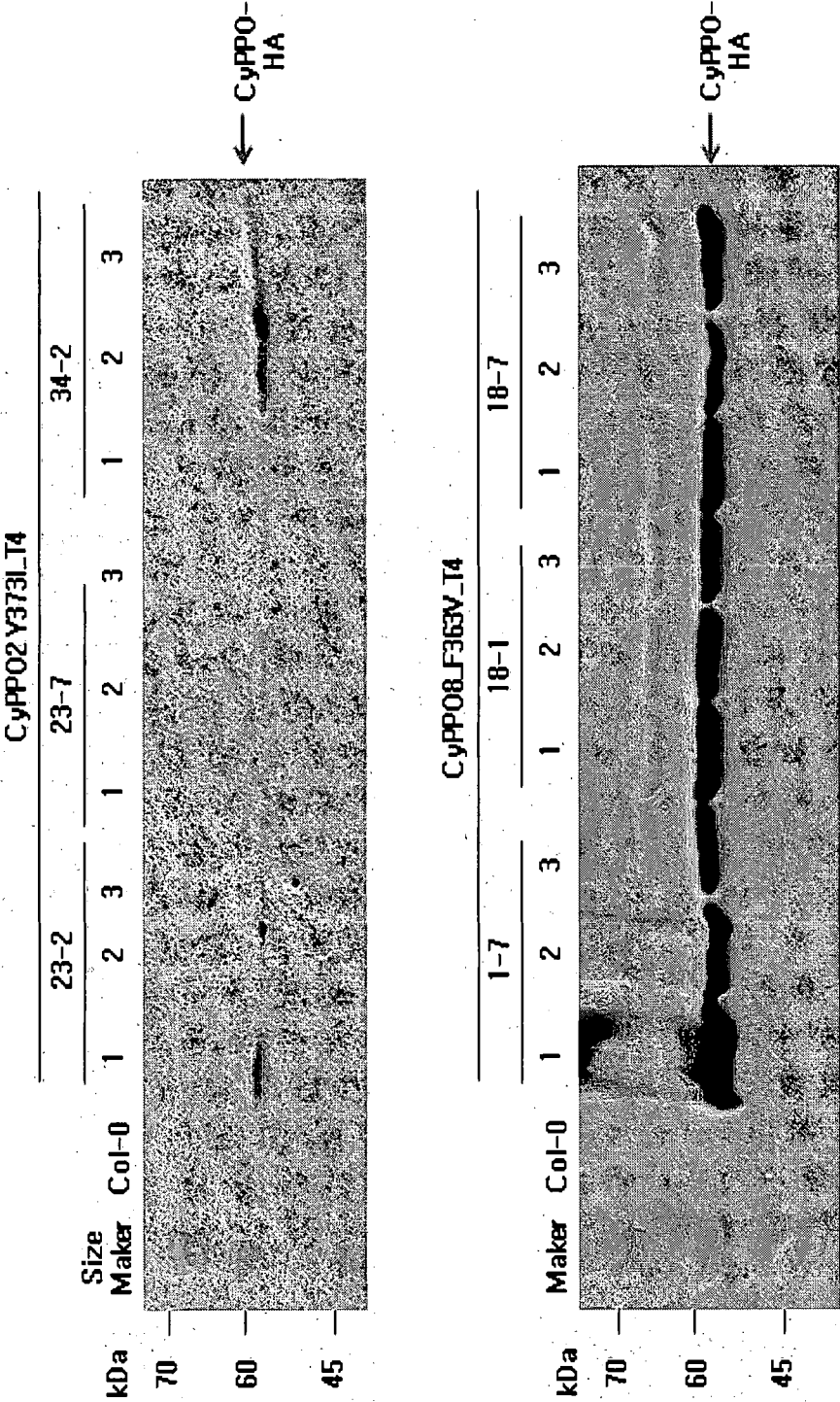
[Fig. 46]



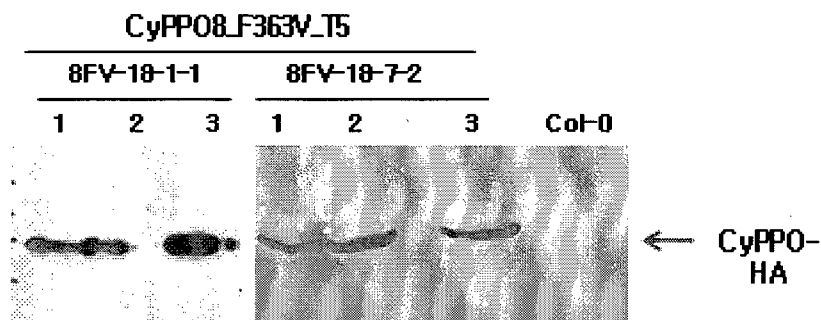
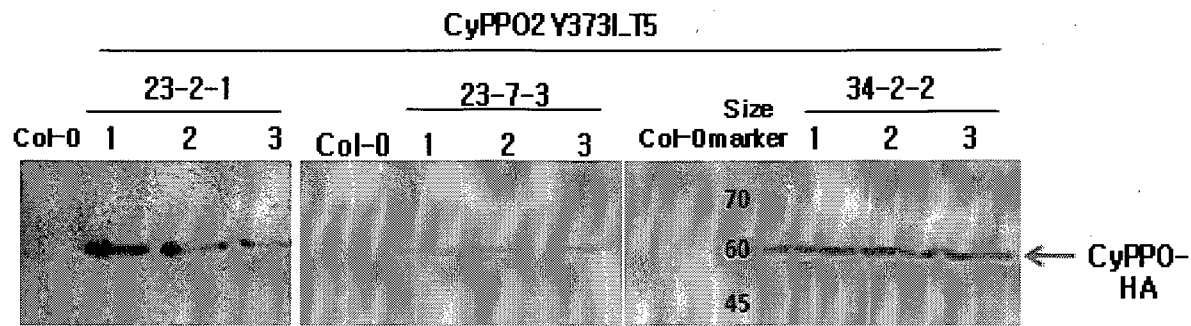
【Fig. 47】



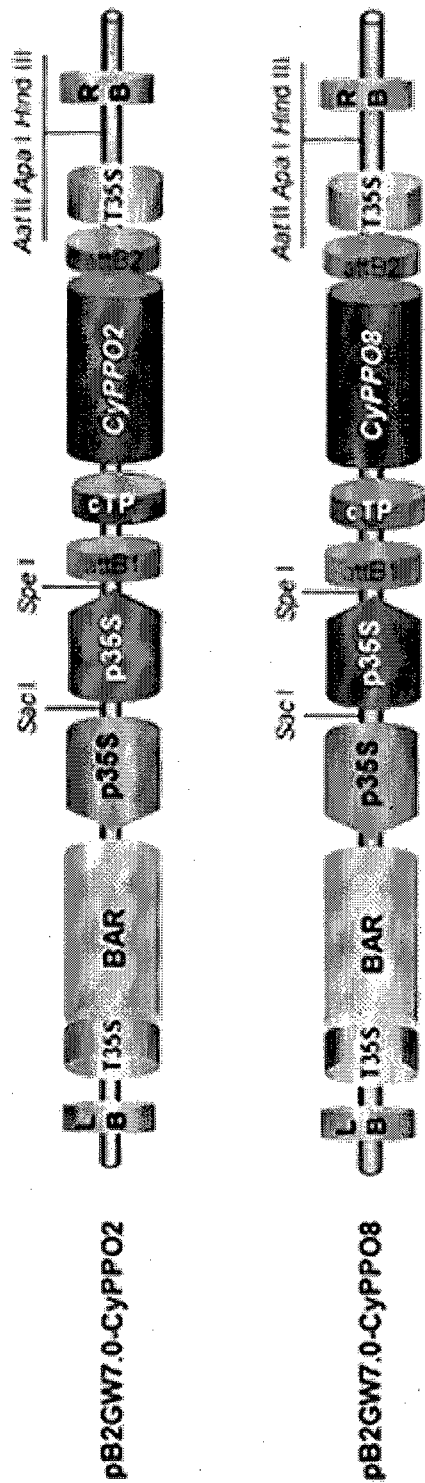
【Fig. 48】



【Fig. 49】



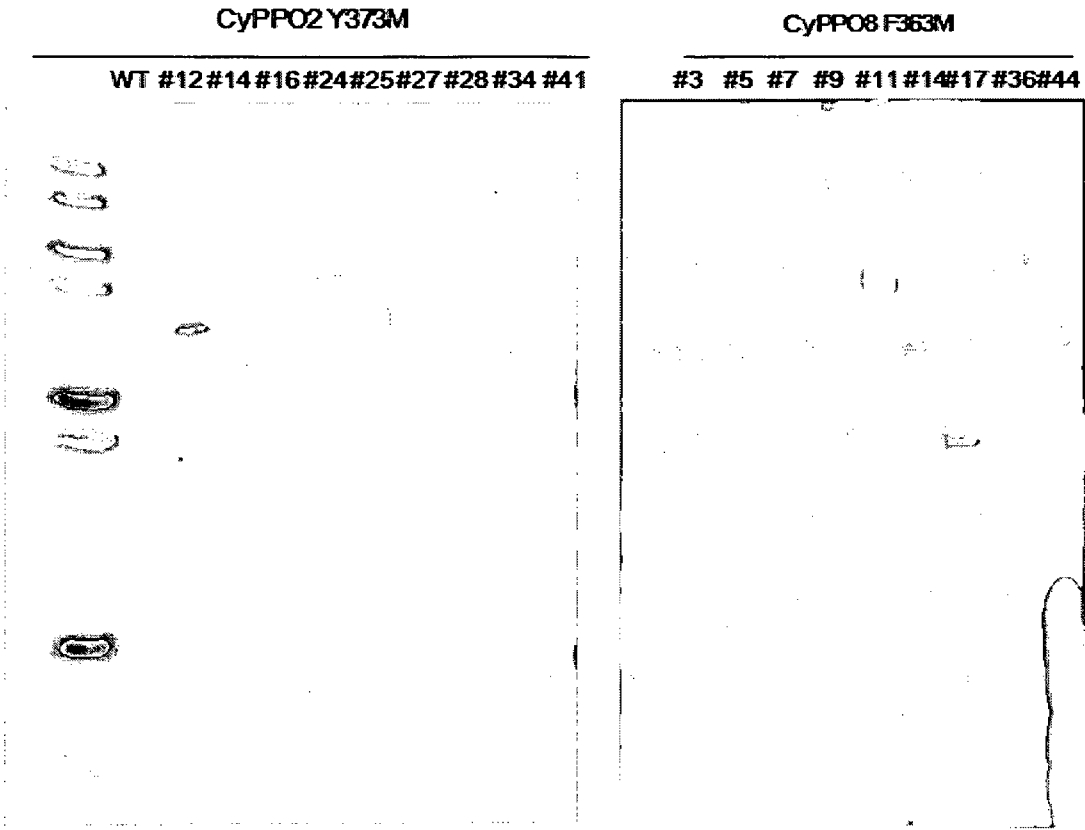
【Fig. 50】



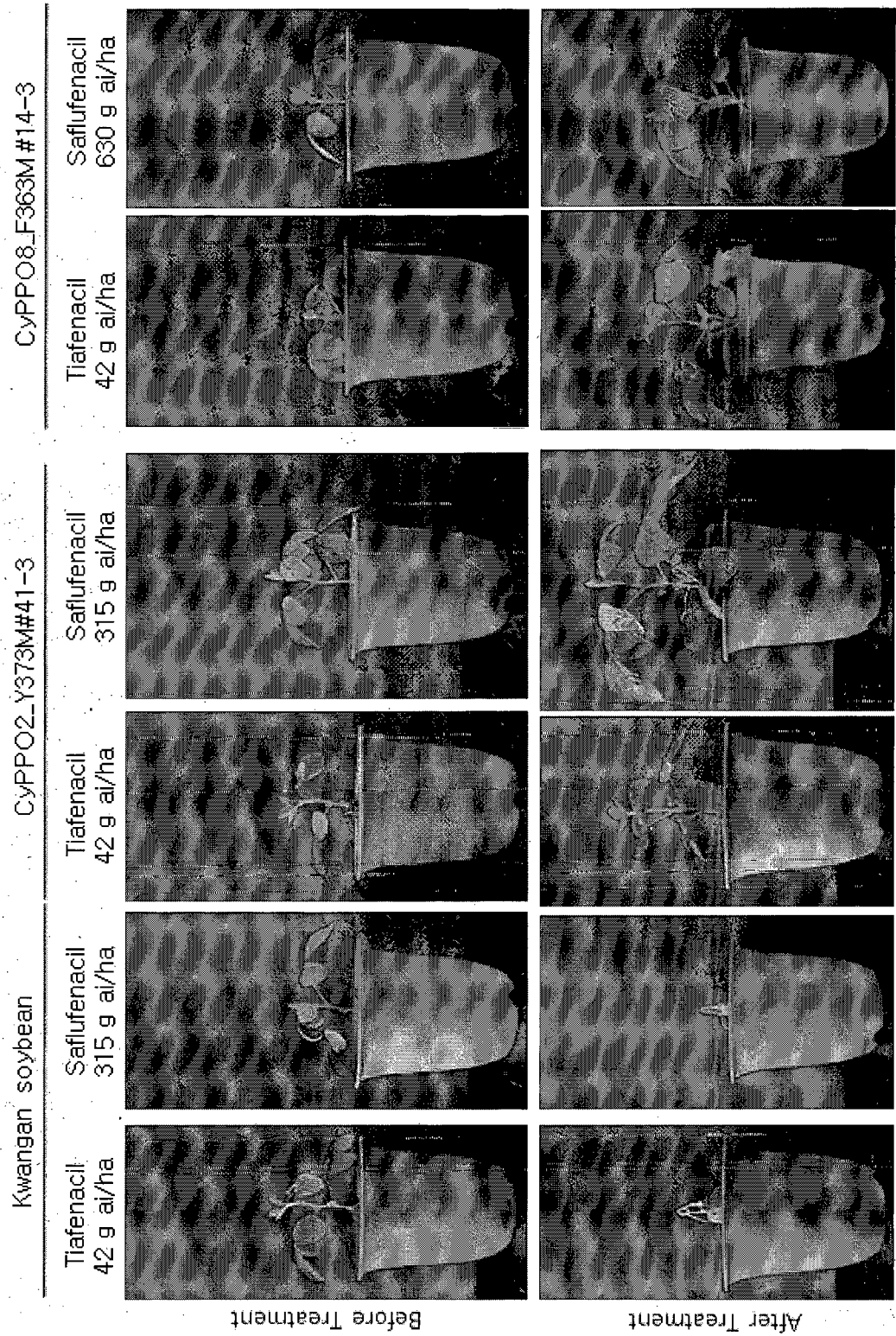
【Fig. 51】

T₀ Transgenic soybean, Cy2 YM

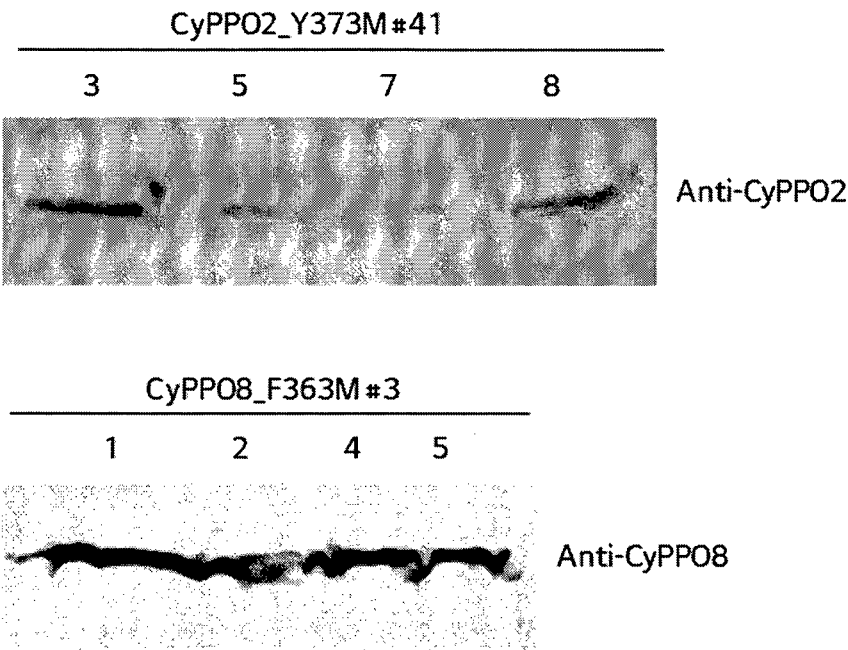
T₁ Transgenic soybean, Cy8 FM



【Fig. 52】



【Fig. 53】



【Fig. 54】

before 10 μ M Tiafenacil treatment

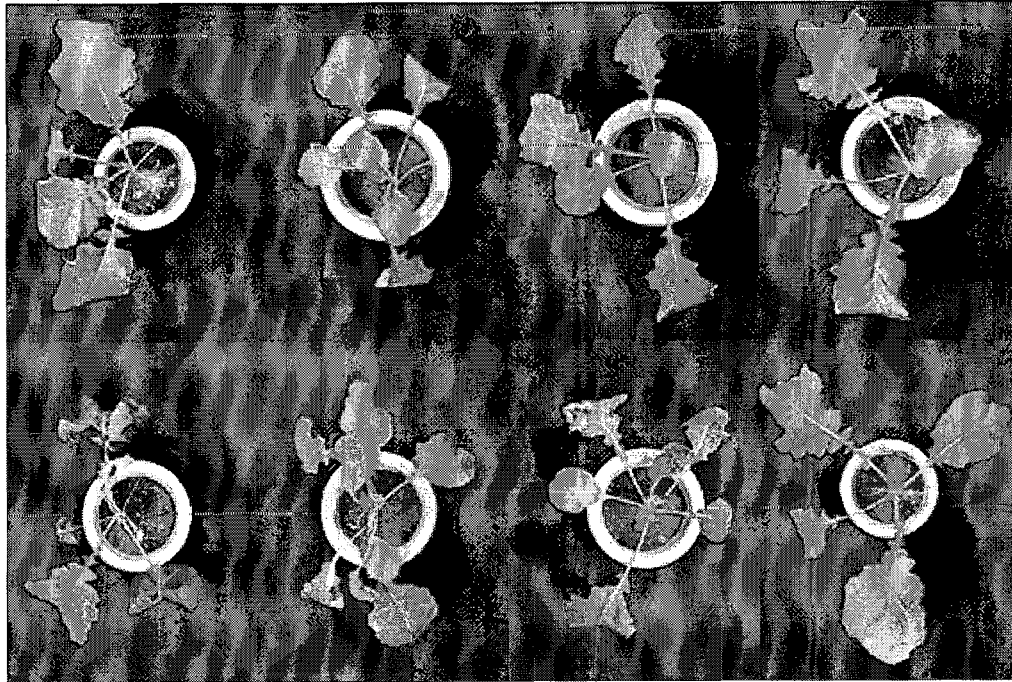
CyPPO8 F363M-2

Youngsan

#4

#7

#8



7 days after 10 μ M Tiafenacil treatment

【Fig. 55】

before 10 μ M Saflufenacil treatment

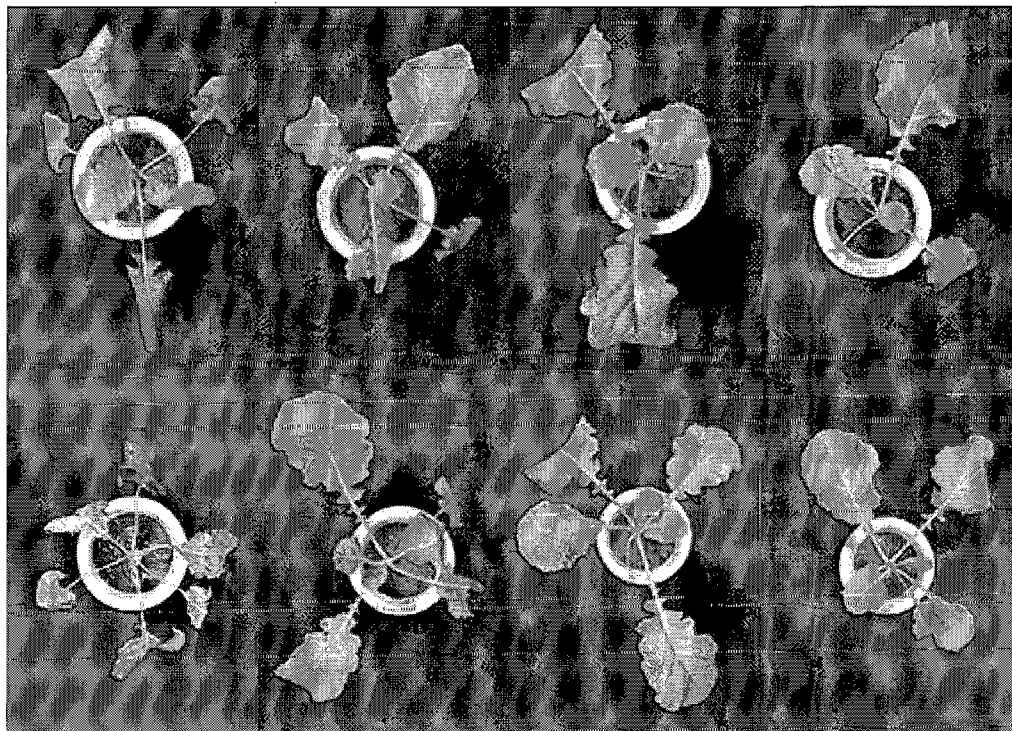
CyPPO8 F363M-2

Youngsan

#6

#9

#10



7 days after 10 μ M Saflufenacil treatment

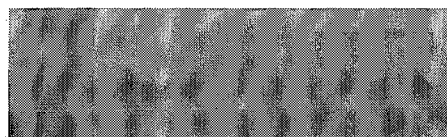
【Fig. 56】

CyPPO8 F363M-2

Youngsan #1 #2 #6 #9 #10



← CyPPO8-HA



A. CLASSIFICATION OF SUBJECT MATTER**C07K 14/195(2006.01)i, C12N 1/12(2006.01)i, C12N 15/63(2006.01)i, C12N 15/82(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K 14/195; C12N 15/82; C12N 9/02; C12N 1/12; C12N 15/63

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: herbicide tolerance, protoporphyrinogen oxidase, mutation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2015-0252379 A1 (HUTZLER et al.) 10 September 2015 See abstract; paragraphs [0047]-[0049], [0056], [0134]-[0137]; Table 1; claims 53, 60, 71, 79.	1-23
Y	NCBI, Reference Sequence no. WP_015177110.1 (19 May 2013) See origin.	1-23
Y	NCBI, Reference Sequence no. WP_009787090.1 (25 May 2013) See origin.	1-23
Y	NCBI, Reference Sequence no. WP_015224927.1 (19 May 2013) See origin.	1-23
A	HAO et al., 'Understanding resistance mechanism of protoporphyrinogen oxidase-inhibiting herbicides: insights from computational mutation scanning and site-directed mutagenesis' Journal of Agricultural and Food Chemistry, Vol.62, pages 7209-7215 (2014) See the whole document.	1-23



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

12 September 2017 (12.09.2017)

Date of mailing of the international search report

12 September 2017 (12.09.2017)

Name and mailing address of the ISA/KR

International Application Division

Korean Intellectual Property Office

189 Cheongsa-ro, Seo-gu, Daejeon, 35208, Republic of Korea

Facsimile No. +82-42-481-8578

Authorized officer

LEE GYONG CHEOL

Telephone No. +82-42-481-8611



INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2017/006276Patent document
cited in search reportPublication
datePatent family
member(s)Publication
date

US 2015-0252379 A1

10/09/2015

AU 2011-342759 A1

13/06/2013

AU 2011-342759 B2

27/04/2017

CA 2818917 A1

21/06/2012

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21/08/2013

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23/10/2013

JP 2014-504855 A

27/02/2014

KR 10-2014-0033330 A

18/03/2014

WO 2012-080975 A1

21/06/2012

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:
YOU ME PATENT AND LAW FIRM

115 Teheran-ro Gangnam-gu Seoul 06134 Republic of Korea

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43bis.1)

Date of mailing
(day/month/year) **12 September 2017 (12.09.2017)**

Applicant's or agent's file reference
OPP173125KR

FOR FURTHER ACTION

See paragraph 2 below

International application No.
PCT/KR2017/006276

International filing date (day/month/year)
15 June 2017 (15.06.2017)

Priority date(day/month/year)
16 June 2016 (16.06.2016)

International Patent Classification (IPC) or both national classification and IPC

C07K 14/195(2006.01)i, C12N 1/12(2006.01)i, C12N 15/63(2006.01)i, C12N 15/82(2006.01)i

Applicant
FARMHANNONG CO., LTD.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43bis. I(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/KR
International Application Division
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon,
35208, Republic of Korea
Facsimile No. +82-42-481-8578



Date of completion of this opinion

12 September 2017 (12.09.2017)

Authorized officer

LEE GYONG CHEOL

Telephone No. +82-42-481-8611



**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.

PCT/KR2017/006276

Box No. 1 Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of :
 - ☒ the international application in the language in which it was filed
 - ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*. I(a))
3. ☒ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13*ter*. I(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*. I(a)).
 - ☐ on paper or in the form of an image file (Rule 13*ter*. I(b) and Administrative Instructions, Section 713).
4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
5. Additional comments:

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/KR2017/006276

Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-23	YES
	Claims	NONE	NO
Inventive step (IS)	Claims	NONE	YES
	Claims	1-23	NO
Industrial applicability (IA)	Claims	1-23	YES
	Claims	NONE	NO

2. Citations and explanations :

Reference is made to the following documents:

D1: US 2015-0252379 A1 (HUTZLER et al.) 10 September 2015

D2: NCBI, Reference Sequence no. WP_015177110.1 (19 May 2013)

※**NOTE:** The phrase “protoporphyrinogen oxidase-5” in claim 22 should be technically considered to be “protoporphyrinogen oxidase”.

1. Novelty and Inventive Step

1.1. Claims 1-4

1.1.1 Claim 1

Claim 1 relates to a polypeptide comprising an amino acid sequence defined in claim 1.

D1, which is considered to be the closest prior art to the subject matter of claim 1, discloses a plant comprising an amino acid sequence encoding a mutated protoporphyrinogen oxidase (mut-PPO) which is resistant or tolerant to a benzoxazinone-derivative herbicide (see abstract; paragraph [0048]; Table 1; claims 53, 60 in D1).

The difference between the subject matter of claim 1 and D1 is that D1 uses a PPO amino acid sequence of *Amaranthus tuberculatus*, whereas the present application uses a modified PPO amino acid sequence of *Oscillatoria nigroviridis*. However, D2 discloses a 486 amino acid sequence of protoporphyrinogen oxidase in the *Oscillatoria nigro-viridis* which is identical to an amino acid sequence of SEQ ID NO:1 in the present invention (see origin in D2).

Continued on Supplemental Box

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/KR2017/006276

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claims 1-4 are not supported by the description as required by PCT Article 6, as its amino acid sequence is broader than justified by the description.

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of : Box No. V

A person skilled in the art would have no particular difficulty in selecting the sequence of PPO in the *Oscillatoria nigro-viridis* of D2 instead of the feature of D1 using the sequence of PPO in the *Amaranthus tuberculatus* for improving resistance or tolerance to herbicide by mutation.

Therefore, claim 1 is novel under PCT Article 33(2) but lacks an inventive step under PCT Article 33(3), as being obvious over D1 in view of D2.

1.1.2. Claims 2-4

As described in point 1.1.1, the additional features of claims 2-4 are merely variations of the disclosure of D1 and D2, and a person skilled in the art would easily arrive at the features of claims 2-4.

Therefore, claims 2-4 are novel under PCT Article 33(2) but lack an inventive step under PCT Article 33(3), as being obvious over D1 in view of D2.

1.2. Claims 5-17

1.2.1. Claims 5-8 and 16

As described in points 1.1.1-1.1.2, claims 5-8 and 16 merely add features which appear to be routine variation to the subject-matter of claims 1-4.

Therefore, claims 5-8 and 16 are novel under PCT Article 33(2) but lack an inventive step under PCT Article 33(3), as being obvious over D1 in view of D2.

1.2.2. Claims 9-15 and 17

The additional features of claims 9-11 would be obvious over D1 disclosing that the benzoxazinone-derivative herbicide can be used in conjunction with protoporphyrinogen-IX oxidase inhibitors which are selected from the group consisting of acifluorfen, fluthiacet, lactofen, proflumazox, and so on (see paragraph [0137] in D1).

The additional features of claims 12-15 would be obvious over D1 disclosing that the plant comprises

Continued on The Next Page

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of : Previous Page

at least one additional heterologous nucleic acid comprising a nucleotide sequence encoding a herbicide tolerance enzyme selected from the group consisting of glyphosate herbicide-tolerant EPSPS, sulfonylurea herbicide-tolerant ALS, and so on (see paragraphs [0056], [0136]-[0137] in D1).

The additional feature of claim 17 corresponds to the feature of D1, considering a transgenic plant cell (see claims 60, 71 in D1).

Therefore, claims 9-15 and 17 are novel under PCT Article 33(2) but lack an inventive step under PCT Article 33(3), as being obvious over D1 in view of D2.

1.3. Claims 18-23

1.3.1. Claims 18-20 and 23

Claims 18-20 and 23 relate to a method of preparing a transgenic plant or algae having herbicide tolerance (claim 18), a method of conferring or enhancing herbicide tolerance of a plant or algae (claim 19), a method of controlling weeds in a cropland (claim 20), and a method of removing an undesired aquatic organism from a culture media (claim 23) using the polypeptide of any one of claims 1 to 4.

As described in points 1.1.1-1.1.2, the polypeptide of any one of claims 1 to 4 would be obvious to a person skilled in the art when D1 and D2 are combined. And D1 discloses a method for controlling undesired vegetation at a plant cultivation site, the method comprising providing, at said site, a herbicide-tolerant plant that comprises a mutated protoporphyrinogen oxidase (mut-PPO); and applying to said site an effective amount of a PPO-inhibiting herbicide (see paragraphs [0047]-[0049]; claims 53, 60, 79 in D1). In view of this, it would be obvious for a person skilled in the art to combine the method of D1 with the sequence of protoporphyrinogen oxidase in the *Oscillatoria nigro-viridis* of D2, thereby arriving at the methods of claims 18-20 and 23.

Therefore, claims 18-20 and 23 are novel under PCT Article 33(2) but lack an inventive step under PCT Article 33(3), as being obvious over D1 in view of D2.

Continued on The Next Page

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of : Previous Page

1.3.2. Claims 21-22

The additional feature of claim 21 would be obvious over D1 disclosing that the benzoxazinone-derivative herbicide can be used in conjunction with one or more other herbicides to obtain control of a wider variety of undesirable vegetation, and the herbicide is applied sequentially with the other herbicide (see paragraph [0134] in D1).

The additional feature of claim 22 would be obvious over D1 disclosing that the plant comprises at least one additional heterologous nucleic acid comprising a nucleotide sequence encoding a herbicide tolerance enzyme, and the herbicide is applied sequentially with the other herbicide (see paragraphs [0056], [0134]-[0137] in D1).

Therefore, claims 21-22 are novel under PCT Article 33(2) but lack an inventive step under PCT Article 33(3), as being obvious over D1 in view of D2.

2. Industrial Applicability

Claims 1-23 are industrially applicable under PCT Article 33(4).

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44bis)

Applicant's or agent's file reference	FOR FURTHER ACTION		See item 4 below
International application No. PCT/KR2017/006276	International filing date (<i>day/month/year</i>) 15 June 2017 (15.06.2017)	Priority date (<i>day/month/year</i>) 16 June 2016 (16.06.2016)	
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237			
Applicant FARMHANNONG CO., LTD.			

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 bis.1(a).

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

- | | | |
|-------------------------------------|--------------|---|
| ☒ | Box No. I | Basis of the report |
| ☐ | Box No. II | Priority |
| ☐ | Box No. III | Non-establishment of opinion with regard to novelty, inventive step and industrial applicability |
| ☐ | Box No. IV | Lack of unity of invention |
| ☒ | Box No. V | Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement |
| ☐ | Box No. VI | Certain documents cited |
| ☐ | Box No. VII | Certain defects in the international application |
| ☒ | Box No. VIII | Certain observations on the international application |

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44bis.3(c) and 93bis.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44bis .2).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland		Date of issuance of this report 18 December 2018 (18.12.2018)
Facsimile No. +41 22 338 82 70		Authorized officer Kihwan Moon
		e-mail: pct.team1@wipo.int

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:
YOU ME PATENT AND LAW FIRM

115 Teheran-ro Gangnam-gu Seoul 06134 Republic of Korea

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43bis.1)

Date of mailing
(day/month/year) **12 September 2017 (12.09.2017)**

Applicant's or agent's file reference
OPP173125KR

FOR FURTHER ACTION

See paragraph 2 below

International application No.

PCT/KR2017/006276

International filing date (day/month/year)

15 June 2017 (15.06.2017)

Priority date(day/month/year)

16 June 2016 (16.06.2016)

International Patent Classification (IPC) or both national classification and IPC

C07K 14/195(2006.01)i, C12N 1/12(2006.01)i, C12N 15/63(2006.01)i, C12N 15/82(2006.01)i

Applicant

FARMHANNONG CO., LTD.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43bis. I(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/KR
International Application Division
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon,
35208, Republic of Korea
Facsimile No. +82-42-481-8578



Date of completion of this opinion

12 September 2017 (12.09.2017)

Authorized officer

LEE GYONG CHEOL

Telephone No. +82-42-481-8611



**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.

PCT/KR2017/006276

Box No. 1 Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of :
 - ☒ the international application in the language in which it was filed
 - ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43bis. I(a))
3. ☒ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13ter. I(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13ter. I(a)).
 - ☐ on paper or in the form of an image file (Rule 13ter. I(b) and Administrative Instructions, Section 713).
4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
5. Additional comments:

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/KR2017/006276

Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-23	YES
	Claims	NONE	NO
Inventive step (IS)	Claims	NONE	YES
	Claims	1-23	NO
Industrial applicability (IA)	Claims	1-23	YES
	Claims	NONE	NO

2. Citations and explanations :

Reference is made to the following documents:

D1: US 2015-0252379 A1 (HUTZLER et al.) 10 September 2015

D2: NCBI, Reference Sequence no. WP_015177110.1 (19 May 2013)

※**NOTE:** The phrase “protoporphyrinogen oxidase-5” in claim 22 should be technically considered to be “protoporphyrinogen oxidase”.

1. Novelty and Inventive Step

1.1. Claims 1-4

1.1.1 Claim 1

Claim 1 relates to a polypeptide comprising an amino acid sequence defined in claim 1.

D1, which is considered to be the closest prior art to the subject matter of claim 1, discloses a plant comprising an amino acid sequence encoding a mutated protoporphyrinogen oxidase (mut-PPO) which is resistant or tolerant to a benzoxazinone-derivative herbicide (see abstract; paragraph [0048]; Table 1; claims 53, 60 in D1).

The difference between the subject matter of claim 1 and D1 is that D1 uses a PPO amino acid sequence of *Amaranthus tuberculatus*, whereas the present application uses a modified PPO amino acid sequence of *Oscillatoria nigroviridis*. However, D2 discloses a 486 amino acid sequence of protoporphyrinogen oxidase in the *Oscillatoria nigro-viridis* which is identical to an amino acid sequence of SEQ ID NO:1 in the present invention (see origin in D2).

Continued on Supplemental Box

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/KR2017/006276

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claims 1-4 are not supported by the description as required by PCT Article 6, as its amino acid sequence is broader than justified by the description.

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of : Box No. V

A person skilled in the art would have no particular difficulty in selecting the sequence of PPO in the *Oscillatoria nigro-viridis* of D2 instead of the feature of D1 using the sequence of PPO in the *Amaranthus tuberculatus* for improving resistance or tolerance to herbicide by mutation.

Therefore, claim 1 is novel under PCT Article 33(2) but lacks an inventive step under PCT Article 33(3), as being obvious over D1 in view of D2.

1.1.2. Claims 2-4

As described in point 1.1.1, the additional features of claims 2-4 are merely variations of the disclosure of D1 and D2, and a person skilled in the art would easily arrive at the features of claims 2-4.

Therefore, claims 2-4 are novel under PCT Article 33(2) but lack an inventive step under PCT Article 33(3), as being obvious over D1 in view of D2.

1.2. Claims 5-17

1.2.1. Claims 5-8 and 16

As described in points 1.1.1-1.1.2, claims 5-8 and 16 merely add features which appear to be routine variation to the subject-matter of claims 1-4.

Therefore, claims 5-8 and 16 are novel under PCT Article 33(2) but lack an inventive step under PCT Article 33(3), as being obvious over D1 in view of D2.

1.2.2. Claims 9-15 and 17

The additional features of claims 9-11 would be obvious over D1 disclosing that the benzoxazinone-derivative herbicide can be used in conjunction with protoporphyrinogen-IX oxidase inhibitors which are selected from the group consisting of acifluorfen, fluthiacet, lactofen, profluzol, and so on (see paragraph [0137] in D1).

The additional features of claims 12-15 would be obvious over D1 disclosing that the plant comprises

Continued on The Next Page

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of : Previous Page

at least one additional heterologous nucleic acid comprising a nucleotide sequence encoding a herbicide tolerance enzyme selected from the group consisting of glyphosate herbicide-tolerant EPSPS, sulfonylurea herbicide-tolerant ALS, and so on (see paragraphs [0056], [0136]-[0137] in D1).

The additional feature of claim 17 corresponds to the feature of D1, considering a transgenic plant cell (see claims 60, 71 in D1).

Therefore, claims 9-15 and 17 are novel under PCT Article 33(2) but lack an inventive step under PCT Article 33(3), as being obvious over D1 in view of D2.

1.3. Claims 18-23

1.3.1. Claims 18-20 and 23

Claims 18-20 and 23 relate to a method of preparing a transgenic plant or algae having herbicide tolerance (claim 18), a method of conferring or enhancing herbicide tolerance of a plant or algae (claim 19), a method of controlling weeds in a cropland (claim 20), and a method of removing an undesired aquatic organism from a culture media (claim 23) using the polypeptide of any one of claims 1 to 4.

As described in points 1.1.1-1.1.2, the polypeptide of any one of claims 1 to 4 would be obvious to a person skilled in the art when D1 and D2 are combined. And D1 discloses a method for controlling undesired vegetation at a plant cultivation site, the method comprising providing, at said site, a herbicide-tolerant plant that comprises a mutated protoporphyrinogen oxidase (mut-PPO); and applying to said site an effective amount of a PPO-inhibiting herbicide (see paragraphs [0047]-[0049]; claims 53, 60, 79 in D1). In view of this, it would be obvious for a person skilled in the art to combine the method of D1 with the sequence of protoporphyrinogen oxidase in the *Oscillatoria nigro-viridis* of D2, thereby arriving at the methods of claims 18-20 and 23.

Therefore, claims 18-20 and 23 are novel under PCT Article 33(2) but lack an inventive step under PCT Article 33(3), as being obvious over D1 in view of D2.

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1.3.2. Claims 21-22

The additional feature of claim 21 would be obvious over D1 disclosing that the benzoxazinone-derivative herbicide can be used in conjunction with one or more other herbicides to obtain control of a wider variety of undesirable vegetation, and the herbicide is applied sequentially with the other herbicide (see paragraph [0134] in D1).

The additional feature of claim 22 would be obvious over D1 disclosing that the plant comprises at least one additional heterologous nucleic acid comprising a nucleotide sequence encoding a herbicide tolerance enzyme, and the herbicide is applied sequentially with the other herbicide (see paragraphs [0056], [0134]-[0137] in D1).

Therefore, claims 21-22 are novel under PCT Article 33(2) but lack an inventive step under PCT Article 33(3), as being obvious over D1 in view of D2.

2. Industrial Applicability

Claims 1-23 are industrially applicable under PCT Article 33(4).