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TITLE: CSR1, The Sole Target of Imidazolinone Herbicide in Arabidopsis thaliana

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ABBREVIATIONS
aaRSs, aminoacyl-tRNA synthetase; ABC, ATP-Binding Cassette; ABRC, Arabidopsis Biological Resource Center; ACO, ACC-oxidase; AHAS, acetohydroxyacid synthase; ALS, acetolactate synthase; AOX, alternative oxidase; aza-dC, 5-aza-2'deoxycytosine; BOA, benzoxazolin-2(3H)-one; FDR, False Discovery Rate; GA, genetically altered; GM, genetically modified; GO, Gene Ontology; GST, glutathione transferase; HB-6, homeobox-leucine zipper protein 6; MATE, multidrug and toxin extrusion; PCD, programmed cell death; RMA, Robust Multi-chip Analysis; ST, steroid sulfotransferase; TC, Transporter Classification.

FOOTNOTES
1In Canada, regulation is based on any novel trait (Tan et al. 2005). Novel trait includes not only genetically modified crops but also all other kinds of genetically altered crops.

ABSTRACT

The imidazolinone tolerant mutant of *Arabidopsis thaliana*, *csr1-2*^{D}, carries a mutation equivalent to that found in commercially available Clearfield® crops. Despite their widespread usage, the mechanism by which Clearfield® crops gain imidazolinone herbicide tolerance has not yet been fully characterized. Transcription profiling of imazapyr (an imidazolinone herbicide)-treated wildtype and *csr1-2*^{D} mutant plants using Affymetrix ATH1 GeneChip® microarrays was performed to further elucidate the biochemical and genetic mechanisms of imidazolinone resistance.

In wildtype shoots, the genes which responded earliest to imazapyr treatment were detoxification-related genes which have also been shown to be induced by other abiotic stresses. Early-response genes included steroid sulfotransferase (ST) and ACC-oxidase (ACO), as well as members of the glycosyltransferase, glutathione transferase (GST), cytochrome P450, ATP-Binding Cassette (ABC) transporter, multidrug and toxin extrusion (MATE), and alternative oxidase (AOX) protein families. Later stages of the imazapyr response involved regulation of genes participating in biosynthesis of amino acids, secondary metabolites, and tRNA.

In contrast to the dynamic changes in the transcriptome profile observed in imazapyr-treated wildtype plants, the transcriptome of *csr1-2*^{D} did not exhibit significant changes following imazapyr-treatment, compared to mock-treated *csr1-2*^{D}. Further, no substantial difference was observed between wildtype and *csr1-2*^{D} transcriptomes in the absence of imazapyr treatment. These results indicate that CSR1 is the sole target of imidazolinone and that the *csr1-2*^{D} mutation has little or no detrimental effect on whole-plant fitness.

KEYWORDS

Imidazolinone Herbicide, CSR1, Branched-Chain Amino Acids Biosynthesis, Food Safety, Affymetrix ATH1 GeneChip® Microarray, *Arabidopsis thaliana*

Introduction

CSR1 encodes a catalytic subunit of an essential enzyme, acetohydroxyacid synthase (AHAS, EC 2.2.1.6) (a. k. a. acetolactate synthase (ALS)), which catalyzes the first step of the synthesis of the branched-chain amino acids, Ile, Leu, and Val (Lee and Duggleby, 2000). AHAS is a target for five classes of herbicides: sulfonylureas, triazolopyrimidines, pyrimidinylthiobenzoates, sulfonylamino-carbonyltriazolinones, and imidazolinones (Tan et al. 2005). The AHAS binding activities of triazolopyrimidines, sulfonylurea, and imidazolinone herbicides have been shown to compete, both with each other, and with endogenous feedback regulation by leucine, a product of AHAS (Subramanian et al. 1991). Based on these observations, Subramanian et al. suggested that the site of herbicide action is the regulatory site of the AHAS protein (1991). Recent 3D structural analysis, however, has shown that sulfonylureas and imidazolinone inhibit AHAS activity by blocking a channel leading to the active site, despite their structural dissimilarity from endogenous AHAS substrates (McCourt et al. 2006).

Some point mutations within *CSR1* retain AHAS activity while conferring herbicide resistance by reducing the herbicide binding capacity of AHAS (Sathasivan et al. 1991; McCourt et al. 2006). Several single-amino-acid-substitution mutations were identified in various plant species, including cotton (*Gossypium hirsitum*), canola (*Brassica napus*), tobacco (*Nicotiana tabacum*), corn (*Zea mays*), *Xanthium strumarium*, *Arabidopsis thaliana*, and yeast (*Saccharomyces cerevisiae*) (Chaleff and Ray, 1984; Haughn et al. 1988; Falco et al. 1989; Bernasconi et al. 1995; Hattori et al. 1995; Rajasekaran et al. 1996; Zhu et al. 1999; Tan et al. 2005). In *A. thaliana,* an amino acid substitution in *csr1-1*^{D} (Pro-197-Ser) results in resistance to the sulfonylurea herbicide chlorsulfuron (Haughn et al. 1988), while amino acid substitutions in *csr1-2*^{D} (Ser-653-Asn)*, csr1-5*^{D} (Ala-122-Thr) and *csr1-6*^{D} (Ala-205-Val) result in resistance to imidazolinone herbicides (Sathasivan et al. 1990; Jander et al. 2003). *csr1-4*^{D} is an intragenic recombination line between *csr1-1*^{D} and *csr1-2*^{D} which is resistant to both chlorsulfuron and imidazolinone herbicides (Mourad et al. 1995). In commercially important species, *csr1* mutants which exhibit imidazolinone resistance have been marketed under the trade name of Clearfield® crops. The commercial success of Clearfield® crops is due, at least partially, to the fact that they are not subject to the same regulations as a genetically modified organism in most countries[1] (Tan et al. 2005).

Substantial equivalence is a widely accepted concept which has had a large influence on the safety legislation of genetically modified (GM) or genetically altered (GA) foods (Kuiper et al. 2001). GM foods are subset of GA foods which includes mutation breeding and somaclonal variation. We use the term “genetically altered (GA)” in place of “genetically modified (GM)” and deal with all types of genetic engineering in an equivalent manner in this article. The rational for this is that a mutation can cause drastic, sometimes unintended, pleiotropic phenotypes encompassing more than just the gene affected directly by the mutation (Schauer et al. 2002; Wurschum et al. 2006). Hence, there is no valid reason to treat GM and other kinds of GA foods differently in terms of food safety. When applying the concept of substantial equivalence to food safety assessment, the food to be approved is compared with foods which have already been approved as safe. If there is no substantial difference, there will be no need for further testing the safety of new food as it is "substantially equivalent" to already approved foods and therefore poses no new risk (Cellini et al. 2004). In case of assessment of GA crops, they are commonly compared with their parental crops for substantial equivalence.

A primary concern about the consumption of GA foods is unintended side-effects, which could be difficult to detect. Unintended off-target effects of chemical substances have also been a major concern in the development and application of medicinal drugs, herbicides, and pesticides (Marton et al. 1998), as well as in the development of GA organisms (Cellini et al. 2004). Thus, the weed management system, which uses combination of a herbicide and a herbicide resistant GA crop, should be assessed for unintended/off-target effects of both the mutation and the herbicide. Microarray analysis using the GeneChip® provides a means by which to detect unintended effects caused by either genetic alteration or by treatment with chemical substances (Stoughton and Friend, 2005; Cellini et al. 2004). This method is especially powerful as it enables simultaneous analysis of transcriptome profiles for virtually all genes in the *A. thaliana* genome, and substantial equivalence for genetic alterations can be examined by comparing GA organisms to their respective parental wildtypes. Off-target effects of chemical substances can be assessed by genetically removing the intended target of the drug/herbicide (Marton et al. 1998). In the case of imidazolinone treatments, *csr1* mutations remove the herbicide target; therefore, comparisons between mock-treated wildtype and mock-treated *csr1* plants will reveal the unintended effects of the genetic

alteration, and comparison between imidazolinone-treated and mock-treated *csr1* plants will reveal the off-target effects of imidazolinone treatment. *csr1-2*^{D} is particularly suitable mutation to this experiment, as no detrimental fitness cost has been observed in this mutant (Roux et al. 2004). The CSR1 protein is believed to be the only target of the herbicide imidazolinone in *A. thaliana*, as a single amino acid mutation can confer imidazolinone tolerance. The possibility of imidazolinone affecting other pathways, however, has not yet been examined. Further, in order to more fully understand off-target effects in imidazolinone-tolerant *csr1-2*^{D} mutants, the effects of the mutation and herbicide must be analyzed together.

Imidazolinone is absorbed through both foliage and root tissues (Tu et al. 2001). After entering a plant, imidazolinone is transported through the xylem and phloem to meristematic tissues where it binds to AHAS and inhibits its activity (Ki = 11 μM for imazapyr) (Chang and Duggleby, 1997). Inhibition of AHAS leads to global elevation of free amino acids level and imbalances in their relative proportions (Höfgen et al. 1995); a relatively standard outcome resulting from inhibition of an enzyme involved in amino acid biosynthesis pathways (Kim et al. 2002). Imidazolinone is a popular herbicide among agricultural producers due to its selectivity and very low toxicity toward fish and animals (Tu et al. 2001). Imazapyr and imazethapyr are two major imidazolinone compounds that have been used in previous studies, and *csr1-2*^{D} exhibits resistance to both of these compounds (Jander et al. 2003).

The biochemical mechanism by which imidazolinone blocks AHAS activity has been studied intensively, but little is known regarding the physiological process by which inactivated AHAS results in plant death. Previously proposed mechanisms describing the lethality of AHAS inhibition have involved unusual accumulation of an intermediate, 2-ketobutyrate and/or 2-aminobutyrate (Shaner and Singh, 1993) or depletion of free branched-chain amino acids pool (Höfgen et al. 1995). Both of these theories have been discredited by empirical data or measurements of free amino acid contents, and the method by which suppression of AHAS activity mediates cell death remains unknown. In this study, we employed the Affymetrix (Santa Clara, CA) *A. thaliana* ATH1 GeneChip® array (Redman et al. 2004) to reveal physiologically-related changes in gene expression

following imidazolinone treatment, and to identify modifications to this process resulting from the *csr1-2*^{D} mutation.

Results

Herbicide Treatment

We employed microarray analysis to identify genes regulated by imidazolinone treatment, the *csr1-2*^{D} mutation and the combination of both. Consistent with previous studies (Roux et al. 2005), the optimal concentration of imazapyr, an imidazolinone herbicide, for this study was investigated and found to be 100 μg L^{-1} (Figure 1). At this concentration, all wildtype plants die while *csr1-2*^{D} mutant plants exhibit 100% survival with no visible damage following 2-weeks of treatment. In order to choose the duration of herbicide treatment, we performed imazapyr time-course studies on both root and shoot (Figure 2). For root growth measurement, four-day-old seedlings were grown vertically on germination medium, transferred to treatment medium, and allowed to grow for up to 60 hours (Figure 2A and S1). Root growth inhibition was observed for imazapyr-treated wildtype within several hours of transfer, while growth inhibition of the shoot took place more gradually. When 7-day-old wildtype seedlings with four leaves (two cotyledons and two true leaves) were transferred to 100 μg L^{-1} imazapyr treatment medium in order to measure shoot growth, the true leaves of imazapyr-treated wildtype expanded a little further for the first few days, but no additional leaves were ever generated (Figure 2B and S1). Imazapyr-treated wildtype seedlings turned yellow after six days and eventually died. No difference was observed between mock-treated and imazapyr-treated wildtype plants over the first two days of treatment; and no difference was observed between mock-treated wildtype, mock-treated *csr1-2*^{D} mutant and imazapyr-treated *csr1-2*^{D} throughout the duration of the treatment.

Based on these data, the Affymetrix ATH1 GeneChip® array analysis was performed on RNA extracted from the shoot tissues of 7-day-old seedlings (wildtype (ecotype: Col-0) and *csr1-2*^{D}) treated with 0 or 100 μg L^{-1} imazapyr for 0, 6, 24 and 48 hours (Table 1). We focused on shoots for this study because the variabilities in root masses resulting from imazapyr-dependent growth inhibition would probably confuse data analysis of RNA extracted from root tissues. The 6- and 48-hour time points were included as early and late regulation time points, respectively, while the 24- and 48-hour

time points were chosen to minimize the influence of circadian rhythm. Mock-treated control seedlings were treated in the same way as imazapyr-treated seedlings with imazapyr-free treatment medium.

Microarray Analysis

Data obtained by microarray were normalized by Robust Multi-chip Analysis (RMA) (Millenaar et al. 2006) and quality control was performed on raw and normalized data using the Affy and AffylmGUI package of Bioconductor (Gentleman et al. 2004; Wettenhall et al. 2006) (Figure S2, S3 and S4). Signal intensities were transformed to log2 by RMA normalization. There were no apparent problems with the MA plots; however, the intensity distribution of one slide, mt6CntC, was substantially different from the others for unknown reasons. Although no fundamental difference in results was observed when excluding data from the mt6CntC slide (Table 2), all remaining normalization and quality control were performed using the remaining 41 slides (Figure S3 and S4), and all data presented here are the aggregate data from these 41 slides unless otherwise noted. An excel tool to display graphs with RMA-normalized values was created to provide a visual aid for the data (Figure S5 and Supplemental Material 1). Ten pair-wise comparisons were performed with moderated Student's t-test using AffylmGUI in order to determine: 1. which genes were induced or suppressed by imazapyr treatment in wildtype (Table 2, A-C); 2. which genes were induced or suppressed by imazapyr treatment in *csr1-2*^{D} (Table 2, D-F); and 3. which genes were differently expressed between wildtype and *csr1-2*^{D} under optimal growth condition (i.e. without treatment) (Table 2, G-J). P-value is adjusted for multiple testing with Benjamini-Hochberg's False Discovery Rate (FDR) (Benjamini and Hochberg, 1995). Tests for differences were also performed with no control of FDR. Such tests are expected to show spurious differences at a frequency related to the number of non-correlated tests that are performed. These tests did detect differences in comparisons related to non-target effects of the chemical (i.e. comparisons Table 2, D-F) and non target effects of the mutation (i.e. comparisons in Table 2, H-K) but the rate of detection was approximately the same or below the expected rate of false discovery (data not shown).

Responses to Imazapyr in Wildtype

Following imazapyr treatment, a total of 1645 (7.21 %) genes were found to be up- or down-regulated by more than 2-fold ($P < 0.05$) at at least one time point in response to imazapyr treatment (Table 2, S1 and Figure 3). Following six hours of treatment, only eight genes were found to be up-regulated, while no genes were found to be down-regulated. These results agree with the physiological observation that the relatively slow process of shoot growth inhibition became apparent only after three days of treatment (Figure 2B). After 24 hours of imazapyr treatment, 382 genes were up-regulated and 114 genes were down-regulated. By 48 hours of imazapyr treatment, 1001 genes were up-regulated and 597 genes were down-regulated. Of the 496 genes regulated at 24 hour, 47 genes were transiently regulated only at this time point. Analysis of a database of Arabidopsis transcription factors (http://datf.cbi.pku.edu.cn/ (Guo et al. 2005)) identified three of these genes as: bZIP transcription factor family protein (At1g42990), homeobox-leucine zipper protein 6 (HB-6, At2g22430) and a putative AP2 domain-containing transcription factor (At1g68550) (Table S2). In addition, 85 transcription factors were found to be up- or down-regulated at later time points.

Genes induced or repressed by imazapyr treatment in wildtype were functionally classified using Gene Ontology (GO) controlled vocabulary (Figure S6, http://arabidopsis.org (Berardini et al. 2004)). Classifications according to subcellular localization and molecular function display high percentage of ribosome components (GO: 0005840; 19.75 % expressed/whole genome) and structural molecule activity (GO: 0005198; 17.93 %), respectively.

No Off-target Effects Detected

In addition to the effect of imazapyr on wildtype seedlings, the effect of the *csr1-2^D* mutation on the transcriptome profile under both imazapyr- and mock-treatment conditions was investigated. In contrast to the global changes in transcriptome profile induced by imazapyr treatment in wildtype shoots, no genes were found to be up- or down-regulated in *csr1-2^D* in response to imazapyr treatment at any time point (Table 2). In other words, the transcriptional regulation induced by imazapyr treatment in wildtype shoots is not observed in *csr1-2^D*, strongly supporting the hypothesis that CSR1 is the sole target of imazapyr. Following analysis of the *csr1-2^D* mutant, the transcriptomes of

mock-treated wildtype and *csr1-2^D* were compared at 0, 6, 24 and 48 hours. Following mock-herbicide treatment, no genes were found to be differentially expressed at any of the time points between wildtype and *csr1-2^D* (Table 2), indicating that the *csr1-2^D* mutation does not affect the transcriptional profile under optimal growth conditions. In other words, the effect of *csr1-2^D* mutation has little or no effect on anything other than changing affinity of AHAS with imidazolinone.

Coordinated Initial Response to Imidazolinone Treatment

Genes which were up-regulated in wildtype shoots following six hours of imazapyr-treatment were hypothesized to be involved in the initial steps of the imidazolinone response. All eight of these genes were also up-regulated at later time points (Table S1). Data mining and expression profiling were performed in attempt to elucidate the function of these genes (Figure S7, http://bbc.botany.utoronto.ca/efp/ (Toufighi et al. 2005)). Interestingly, all eight genes of interest were also regulated by UV-B treatment, and seven were also regulated by NaCl (with DTX4 as the exception) (Figure S7), suggesting that these genes may play roles in general stress responses. Additionally, all eight genes were found to be induced by at least one biotic stress condition and by the protein synthesis inhibitor cycloheximide. In the process of the investigation, it was noticed that the expression profiles of these eight genes are somewhat similar in response to various stimuli. Expression angler (http://bbc.botany.utoronto.ca (Toufighi et al. 2005)) was employed to confirm the correlation among these genes with “AtGenExpress stress set” as the data set searched. It turned out that all of these eight genes show highly similar transcriptional responses in response to abiotic stresses (Table 3).

These eight genes were induced also by other herbicides which target AHAS (Figure 4). Two imidazolinone family herbicides, imazethapyr and imazaquin, and a sulfonylurea family herbicide, chlorsulfuron, were examined in addition to 100 μg/L and 1 mg/L imazapyr. This result supports the finding that induction of these eight genes is the consequence of AHAS inhibition. It also serves as an additional validation of the microarray result. There was no clear indication of imazapyr dosage effect.

UGT74E2 (At1g05680) is a putative indole-3-acetate beta-glucosyltransferase which was induced by sirtinol, a chemical which specifically induces many auxin responsive genes, but not by auxin (Zhao et al. 2003). Kim et al. reported partially contradictory results, in which At1g05680 was up-regulated by dark-treatment, ABA and auxin, as well as in the *phyB* mutant (Kim and von Arnim, 2006). Up-regulation by an allelochemical, benzoxazolin-2(3*H*)-one (BOA), has also been reported (Baerson et al. 2005). Finally, UGT74E2 was also shown to be up-regulated by a cytosine methylation inhibitor, 5-aza-2'deoxycytosine (aza-dC), indicating its role in methylation (Chang and Pikaard, 2005).

Alternative oxidase 1A (*A*OX*1A*) (At3g22370) is expressed in mitochondria and is known to respond to stresses (Clifton et al. 2005; Escobar et al. 2006). Clifton et al. reported co-expression of alternative respiration components in response to stresses; with an especially tight linkage between *NDB2* (At4g05020) and *AOX1A* (Clifton et al. 2005).

AtSOT12 (a.k.a. AtST1 At2g03760) is a brassinosteroid sulfotransferase (ST) which was isolated as a protein produced in respond to salicylic acid, methyl jasmonate and infection by bacterial pathogens (Lacomme and Roby, 1996; Marsolais et al. 2006). *AtSOT12* has also been identified as a NaCl-specific responsive gene exhibiting the largest-fold change (19-fold) in 7-day-old seedlings treated with 100 mM NaCl (Kreps et al. 2002), and as a gene which responds to auxin (1 μM IAA) and brassinosteroid (10 nM brassinolide) treatments (Goda et al. 2004). The biological function of this protein, however, has not yet been characterized.

At5g43450 is an ACC-oxidase (ACO) whose expression is induced by ethylene treatment in wildtype, but not in the ethylene-insensitive mutant, *ein2-1* (Vandenbussche et al. 2003; De Paepe et al. 2004). At5g43450 is also induced by NO treatment (Parani et al. 2004) and the *rcd1-1* mutation (Ahlfors et al. 2004). Six other genes induced by imazapyr treatment namely At2g21640, At3g08590, At3g22370 (*AOX1A*), At3g27060, At3g45730 and At3g50970 (*XERO2*) are among 15 genes with significantly altered expression patterns in *rcd1-1*. Interestingly, some of these genes are up-regulated by imazapyr but down-regulated in *rcd1-1* or *vice versa* (Table S1 (Ahlfors et al. 2004)).

CYP81D8 (At4g37370) is a cytochrome P450. It is induced by dark-treatment, ABA and auxin and cytokinin (Kim and von Arnim, 2006).

DTX4 (At2g04040) is a multidrug and toxin extrusion (MATE) transport protein which is induced by BOA (Brown et al. 1999; Li et al. 2002; Baerson et al. 2005).

ATGSTU9 (At5g62480) is an uncharacterized glutathione *S*-transferase (GST) (Wagner et al. 2002).

No literature was found regarding the function of At2g41730. It is classified as a "mitochondrial protein" (GO: 0005739) by the GO Cellular Component annotation based on computer simulation and was induced by low oxygen (Klok et al. 2002).

Detoxification Is the Initial Response

Following identification of the eight early responsive genes described above, 67 genes up- or down- regulated by imazapyr at later time points were found to share high Pearson's correlation coefficient with those eight genes (Table 3). This suggests that the eight early-response and the 67 later-response genes are functioning in the same pathway, and that this pathway may be common to both imazapyr treatment as well as several other abiotic stresses.

Publicly available datasets for some herbicide treatments and AtGenExpress stress series (Craigon et al., 2004), were obtained in order to compare the transcriptional profile in response to imazapyr to that of other kinds of herbicides and abiotic stresses (Figure S8 and Table S3). Three herbicides with different modes of action, 2,4-dichlorophenoxyacetic acid (2,4-D) which is a synthetic auxin, isoxaben which inhibits cellulose synthesis and norflurazon which inhibits carotenoid biosynthesis, were compared and only norflurazon appeared to share some transcriptional regulation. Out of eight abiotic stress series microarrays, salt, UV-B and cold seem to share common pathways as well.

Further investigation was performed to determine the pathway in which these genes are functioning. The total of 75 genes which share high Peason's correlation coefficient include those for glycosyltransferases (Bowles et al. 2006), GSTs (Dixon et al. 2002), cytochrome P450s (Morant et al. 2003; Baerson et al. 2005), ATP-Binding Cassette (ABC) transporters (Geisler and Murphy, 2006) and MATE efflux family proteins (Diener et al, 2001). All of these genes have been hypothesized to function in

detoxification (Schaeffner et al. 2002; Pilon-Smits, 2005). In additon to these genes, this group also included AOXs, which have been implicated in stress adaptability (Arnholdt-Schmitt et al. 2006). Taken together, these data indicate that the first response towards imazapyr treatment is the induction of detoxification-related genes. To investigate this further, members of the glycosyltransferase, GST, cytochrome P450, ABC transporter, MATE, AOX, ST and ACO gene families were tested against imazapyr-regulated genes in order to examine which families are involved in the detoxification of imazapyr (Table 4).

Lists of genes were obtained from the TAIR web site (http://www.arabidopsis.org/browse/genefamily/) for glycosyltransferase, GST, cytochrome P450, ABC Superfamily/ABC transporter family members; and from the plantsT web site (http://plantst.genomics.purdue.edu/ (Tchieu et al. 2003)) for ABC Superfamily (Transporter Classification (TC): 3.A.1) and MATE family proteins (TC: 2.A.66). A list of the components in the alternative respiratory pathway and ST gene family was prepared according to the literature (Klein and Papenbrock, 2004; Clifton et al. 2005). A list for ACO genes was prepared based on De Paepe et al (De Paepe et al. 2004) and GO annotation with keyword "1-aminocyclopropane-1-carboxylate oxidase activity" (GO: 0009815). Among a total of 5669 genes listed at the TAIR web site under the selected gene families which were also represented in the ATH1 array, 355 (6.26%) of the genes were up- or down-regulated in response to imazapyr (Table S4). A high percent of the observed up- or down-regulated genes were members of the alternative respiratory pathway and GST gene families (Table 4 and 5). In contrast, only one gene each from the ST and ACO families was induced (Table 4 and 5), indicating the distinct role of members of these two gene families in the imazapyr response.

The Pathways Affected by Imazapyr Treatment

In addition to the microarray screens above, we selected pathways likely to be involved in the imazapyr response for further investigation. The metabolic pathway producing branched-chain amino acids was an obvious choice, as CSR1 is a catalytic subunit of AHAS, the first enzyme in branched-chain amino acid synthesis. Inhibition of AHAS results in an altered amino acid profile (Höfgen et al. 1995). We therefore hypothesized that the expression of enzymes involved in the biosynthesis of other amino

acids would be altered following imazapyr treatment. An altered amino acid profile may interfere with the production of tRNA, proteins enriched in branched-chain amino acids, ribosomes, and secondary metabolites derived from amino acids. Further, as altered amino acid profiles may lead to amino acid starvation, plants may up-regulate the expression of genes encoding amino acid transporters, in order to relocate amino acid and other forms of nitrogen after the application of imazapyr. Under optimal growth conditions, the majority of cellular nitrogen is located in chloroplasts (Hortensteiner and Feller, 2002). Nitrogen relocation involves protein degradation in the chloroplast and the subsequent transport of amino acids to other parts of the plant. Both amino acid and peptide transporters are important players in rapid redistribution of nitrogen source (Stacey et al. 2002). The death of the plant after imazapyr treatment is believed to be similar to senescence, with the caveat that cell death is not programmed, but rather the result of an external stimulus. The expression of genes involved in developmental regulation may also be altered following herbicide treatment, as inhibition of growth was observed at later stages of the imazapyr response.

A list of genes involved in the predicted pathways described above was prepared and compared with the list of imazapyr responsive genes (Table 6 and 7). The genes involved in the amino acid biosynthesis pathways were downloaded from AraCyc, a biochemical pathways database for *A. thaliana* (http://www.arabidopsis.org/biocyc/ (Mueller et al. 2003)). Out of 4645 Aracyc entries (1609 genes) represented in ATH1 array, 499 (10.74 %) entries (162 (10.07 %) genes) were up- or down-regulated by imazapyr treatment (Table S5).

Of the 29 genes involved in branched-chain amino acid biosynthesis, only one gene, 3-ISOPROPYLMALISOM-RXN (At2g05710), displayed significant induction by imazapyr in wildtype but not in resistant *csr1-2*^{D} mutant shoots (Table 6 and 7). Although some of the genes including *CSR1* (At3g48560, which did not reach the cut off value) displayed small degrees of induction or repression (Figure S9), transcriptional regulation may not be a major regulatory mechanisms of branched-chain amino acids synthesis. Out of the 177 amino acid biosynthetic genes investigated, 16 were up-regulated and one was down-regulated by imazapyr treatment. These genes are involved in synthesis of 10 out of 20 amino acids (Table 6 and 7), and their regulation following imazapyr treatment

demonstrates that the effects of AHAS inhibition are not limited to branched-chain amino acid synthesis.

The list of aminoacyl-tRNA synthetase (aaRSs), enzymes which catalyze the addition of an amino acid to a tRNA, was obtained from Duchen et al. (2005). Nine out of 48 aaRSs were up-regulated and one was down-regulated following imazapyr treatment. A list of ribosomal proteins was obtained from the GO functional classification (GO: 0005840; Figure S6). Expression of 80 out of 312 ribosomal proteins represented in the ATH1 arrays was altered after imazapyr treatment, indicating that herbicide treatments alter protein synthesis. There was no list of BCAA rich genes available for analysis.

Secondary metabolites of amino acids include auxin, pigments such as anthocyanines, defensive phytoalexins, bioactive alkaloids, and structural lignin. A list of genes involved in these pathways was obtained from the Aracyc website (Table 6 and S5). Some of the genes involved in the synthesis of secondary metabolites show altered transcription following imazapyr treatment, supporting the hypothesis that inhibition of CSR1 triggers a global change by altering amino acid levels.

A data set of amino acid (TC: 2.A.3 and 2.A.18) and peptide transporter (TC: 2.A.17, 2.A.67 and 9.B.12) genes was obtained from the PlantsT website (http://plantst.genomics.purdue.edu/) (Tchieu et al. 2003). Regulation of only one gene each out of the 56 amino acid and 60 peptide transporters represented on the ATH1 microarray was observed. These data indicate that alteration of the transcriptional regulation of amino acid and peptide transporters may not be part of the imazapyr response.

Some senescence-related genes ("leaf senescence", GO: 0010150 (van der Graaff et al. 2006)) were up- or down-regulated by imazapyr treatment (Table 5). However, the frequency of regulation was not very high. Additionally, transcription of senescence marker genes, such as SAG12 (At5g45890; Lohman et al., 1994), SEN1 (Oh et al., 1994) and YLS4 (Yoshida et al., 2001), was not altered substantially by imazapyr. Thus, the regulation of some senescence related genes by imazapyr treatment is not an indication of senescence, but instead probably the result of the activation of other pathways which

share some proteins with senescence. Neither programmed cell death (PCD) nor aging is likely to be the part of imazapyr response as low percentage each of genes related to death (GO: 0016265) and aging (GO: 0016280) were regulated by imazapyr (Table 5).

Discussion

Time course analysis of transcriptome profiles in imidazolinone-sensitive (wildtype) and imidazolinone-resistance (*csr1-2*^{D}) genotypes has demonstrated coordinated regulation of several pathways following the inhibition of AHAS by imazapyr treatment. Additionally, this study has demonstrated that there is no significant "baseline difference" between the transcriptomes of wildtype and *csr1-2*^{D} shoots under optimal growth conditions. In other words, *csr1-2*^{D} and wildtype plants are "substantially equivalent". Therefore, mutations equivalent to *csr1-2*^{D} mutation in crop plants are not likely to present any risks to consumers. This result is consistent with a previous phenotypic study conducted by Duggleby's group, which reported no fitness cost for *csr1-2*^{D} (Roux et al. 2004). Further, imazapyr treatment acts solely by inhibiting CSR1. All of the changes caused by imazapyr treatment, including: global transcriptome expression, growth inhibition, and eventual plant death are all caused by the inhibition of CSR1 function. Thus, all these events are the consequence of an inhibition of branched-chain amino acid biosynthesis. No off-target effects were detected following 100 μg/L imazapyr application. The concentration of imazapyr is lower than typical field application; however, herbicide applied in the field is subject to dilution and therefore should not be compared to *in vitro* application where herbicide concentration will be maintained. The fact that neither the *csr1-2*^{D} mutation nor imazapyr treatment causes off-target effects strongly support the safety of the imidazolinone-Clearfield® herbicide management system. Even though transcriptional regulation is not the only way of regulation, result of those regulations (e. g. protein modification) most likely to affect transcriptome profile.

Sequence of Events Following Imazapyr Treatment

Chronologically, one of the earliest responses to imazapyr treatment is the inhibition of root growth, which occurs several hours after herbicide application (Figure 2 and 5). In shoots, the transcriptional induction of eight genes was observed after six hours of treatment, a time point when there was no visible shoot phenotype observed (Figure 2

and Table 3, S1). None of these eight genes was a transcription factor; they instead encoded components of detoxification mechanisms which are common to other abiotic stress responses. While the regulation of detoxification genes persists throughout the time course of imazapyr treatment, other responses also emerge at later time points. These responses include: an alteration in biosynthesis of amino acids and their secondary metabolites and induction of tRNA biosynthesis.

Our microarray data indicate that neither induction of senescence nor programmed cell death (PCD) plays a major role in the response to imazapyr. Thus, imidazolinone likely induces plant death by different mechanisms than these two processes. However, the observed lack of response by senescence- and PCD-related genes could simply be because our data set focused primarily on earlier time points, to the exclusion of later events. As the imidazolinone response is a relatively slow process, the possibility that the regulation of genes involved in senescence or PCD is involved in this process at a later stage cannot be dismissed.

Newly-Found Components of Detoxification Pathways

Through microarray analysis, the coordinated regulation of detoxification related genes in response to imidazolinone in wildtype shoot has been revealed. Although many of the genes involved had previously been established to play a role in the detoxification process, our analysis has revealed some genes not previously implicated in detoxification. These genes include *AtSOT12* (AtSOT12 (At2g03760), At2g41730, and components of alternative respiratory pathway. Furthermore, co-expression of three tandemly repeated MATE transport proteins, namely *DTX4* (At2g04040), *DTX3* (At2g04050) and *DTX1* (At2g04070), was also observed. These three MATE transport proteins have high homology to one another (Li et al. 2002). Co-expression of this tandemly-repeated MATE transport protein cluster has been proposed as being analogous to a bacterial abiotic resistance mechanism which functions by amplifying genes to a high copy number of transcripts when the cells encounter abiotic stress (Diener et al. 2001)

Future study

This study has shown that genes involved in the adaptation to imidazolinone also function in other abiotic stress responses. This raises the possibility that other kinds of stress may interfere with the efficiency of imidazolinone treatment. Abiotic stress and imidazolinone treatment may have additive effects and result in plant death at lower concentrations of imidazolinone. However, there is also the possibility that the higher basal expression levels of stress-related gene resulting from abiotic stresses may “boost” the imidazolinone adaptation process and grant plants increased tolerance towards the herbicide application.

The system of imidazolinone treatment and imidazolinone-resistant *csr1-2*^{D} mutant combination used in this study can also be used as a model for studying abiotic stresses such as salinity and heavy metal stresses, as they share common detoxification pathway. One advantage to this system is that imidazolinone is not likely to be found under natural conditions, unlike metal ions or osmolites. The high specificity of the imidazolinone response also facilitates simpler interpretation of experimental results.

Materials And Methods

Plant Materials and Growth Conditions

Seeds of *csr1-2*^{D} (ecotype: Col-0), were obtained from The Arabidopsis Biological Resource Center ((ABRC) Columbus, Ohio, USA) stock center. Seeds were surface-sterilized first with 70% (v/v) ethanol for a brief period, then with sterilization solution (20% (v/v) Clorox® (The Clorox Company, California, USA), 0.05% (v/v) Triton-X 100 (Fisher Scientific, Hampton, New Hampshire, USA)) for about 15 minutes. Seeds were then rinsed three times with distilled water. Surface-sterilized seeds were kept at 4°C for a minimum of three days for stratification. Before sowing, seeds were suspended in 0.15% (w/v) Agarose (FMC BioProducts, Hercules, California, USA).

For primary root measurement, seeds were sown on a cellophane membrane (Bio-Rad, Hercules, California, USA) placed on germination medium (1/2 X MS salts (Sigma, St. Louis, Missouri, USA) (pH 5.7), 3% (w/v) Sucrose, 1.2% (w/v) Gibco Phytagar (Invitrogen, Carlsbad, California, USA) as previously described (Zhu et al. 2002). For all other experiments, seeds were sown directly on germination medium with 0.6% (w/v) Phytagar instead of 1.2%.

Herbicides treatments were performed by transferring the plants to treatment medium supplemented with imazapyr (American Cyanamid Company CL243.997, currently BASF, Ludwigshafen, Germany), imazethapyr (American Cyanamid Company AC263.499), imazaquin (American Cyanamid Company CL252.214), or chlorosulfuron (Chem Service, West Chester, PA). Concentration of imazapyr is 100 μg L^{-1} (30 mg AI ha^{-1}) unless it is specifically noted. Filter-sterilized herbicides were added into the media after autoclaving when the temperature was less than 50°C.

Growth Measurements

For the measurement of shoot fresh weight, shoots were excised and weighed using Mettler AK160 (Mettler Instrumente AG, Zurich, Switzerland). For the measurement of root growth, the position of the primary root tips were marked at the bottom of Petri dishes at the time of transfer and scanned by a flat bed scanner (AGFA Duoscan T1200, Mortsel, Belgium) at 300 pixels in^{-1} after the treatments. The scanned images were saved

as TIF format and were measured using NIH's Scion Frame Grabber as described (Buer et al. 2000). For the leaf number measurement, 7-days-old seedlings with four leaves (Two cotyledons and two true leaves) were transferred to treatment media and leaf numbers were counted under microscope each day. These experiments had been performed at least three times and consistent results had been obtained each time.

RNA Extraction and Bioanalyzer Analysis

7-day-old seedlings were treated with 100 μg L^{-1} imazapyr for 0, 6, 24 and 48 hours in the same way as shoot growth measurement experiments for microarray analysis and with 100 μg L^{-1} or 100 mg L-1 imazapyr, 200 μg L^{-1} imazethapyr, 200 μg L^{-1} imazaquin or 200 μg L^{-1} chlorosulfuron for 48 hours. After the treatment, seedlings were placed on the wet filter paper for excision of the shoots. Shoots from 30 seedlings were pooled for RNA extraction. Each pool of 30 plants was harvested from a single Petri dish which was placed randomly in a growth cabinet to minimize the positional effects.

RNA extraction was performed using RNeasy Plant Mini Kit (Qiagen, Germantown, Maryland, USA) according to the manufacturer's protocol. After the extraction, RNA quality was assessed using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) according to the manufacturer's protocol.

Microarray Analysis

cRNA synthesis, hybridization and scanning were performed at Botany Affymetrix GeneChip Facility, University of Toronto (Toronto, Ontario, Canada). Data were normalized by Robust Multi-array Analysis (RMA) (Irizarry et al. 2003) using R (http://www.r-project.org/)-Bioconductor (http://www.bioconductor.org/) (Gentleman et al. 2004) software with affylmGUI package (Wettenhall et al. 2006). Per slide MA plots were generated by affy package (Gentleman et al. 2004) and lists of differentially expressed genes were generated by affylmGUI.

For analysis of herbicide and stress-related datasets are obtained from NASC (http://affymetrix.arabidopsis.info/) as CEL file formats provided through Affywatch CD/DVD subscription (The Nottingham Arabidopsis Stock Centre, Sutton Bonington, UK). NASCArrays Experiment Reference Number are 352 for 2,4-D, 27 for isoxaben, 89

for norflurazon, and 137 to 146 for AtGenExpress stress series Only shoot slides are used for analysis of AtGenExpress stress series. These data were normalized and analyzed in same way as imazapyr datasets. Normalization was performed on all the time points and comparison was made only on slides for 24 hours of treatment.

Time-course expression profiles of each gene were created using a Visual Basic application in Microsoft Excel. Expression values were RMA-normalized with 41 slides except for mt6CntC, and then averaged for each treatment group. Error bars were computed to indicate standard deviation among triplicates (duplicate for *csr1-2*^{D}/control/6h as mt6CntC is removed). A ZIP compressed Excel file containing a macro to produce expression profiles of any gene analyzed in this study is provided as a supplemental material.

Reverse Transcriptional (RT) PCR

cDNA synthesis was performed using 1mg of RNA template, super script III reverse transcriptase (Invitrogen, Carlsbad, California, USA) and oligo dT_{20} primer according to manufacture's protocol. 25 cycles of PCR (1 min at 94°C, 1 min at 60°C, and 1 min at 72°C) was performed following cDNA synthesis. Primer sets used are At1g05680_F (TCCAATGTCCCAGTTCTGCAAA) and At1g05680_R (TTACAATAAGTCCTTTTTCACCGA) for *UGT74E2*, AOX1A_F (TGATGATAACTCGCGGTGGAGC) and AOX1A_R (GTCGAAGCGATTTGCAGTGTAGTA) for *AOX1A*, ST_F (CGATCTCTCAAGTCTCAACAATG) and ST_R (GGCAATTTCCCTTCTTTGTTAACT) for *AtSOT12*, At5g43450_F (AACGATCTCACGACTTTTATCTCG) and At5g43450_R (GAGGGACATCGACCCAATAATCTT) for At5g43450, CYP81D8_F (GGAAACCAAAACCCTAATTTTCTC) and CYP81D8_R (GATGGTTCAACACGTTCGACAA) for *CYP81D8*, DTX4_F (GACCATTGCTCAGTACTTATTGCC) and DTX4_R (TTCCCAGCTCCCAAATTGTTTG) for *DTX4*, AtGSTU9_F (AGTGATACTCCATGGATCATTCGC) and AtGSTU9_R (AAGTTTCAAGAACGGGAGAGAGAG) for AtGSTU9, and At2g41730_F

(AAGGTCGTCACCAAGGCATCGTAA) and At2g41730_R (TGTTCATCATGATCTTCACCACA) for At2g41730, and Tub_F (GGGCTAAAGGACACTACACTGAAGG) and Tub_R (CCTCCTGCACTTCCACTTCGTCTTC) for $-tubulin (TUB8; AT5G23860).

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Table1. Experimental design for microarray

Treatment	Genotype \ Time (h)	0	6	24	48
Control	**Wildtype (Col-0)**	WT0CntA WT0CntB WT0CntC	WT6CntA WT6CntB WT6CntC	WT24CntA WT24CntB WT24CntC	WT48CntA WT48CntB WT48CntC
	csr1-2^D	mt0CntA mt0CntB mt0CntC	mt6CntA mt6CntB mt6CntC*	mt24CntA mt24CntB mt24CntC	mt48CntA mt48CntB mt48CntC
Imazapyr	**Wildtype (Col-0)**	**N/A**	WT6ImA WT6ImB WT6ImC	WT24ImA WT24ImB WT24ImC	WT48ImA WT48ImB WT48ImC
	csr1-2^D	**N/A**	mt6ImA mt6ImB mt6ImC	mt24ImA mt24ImB mt24ImC	mt48ImA mt48ImB mt48ImC

* mt6CntC was omitted from analysis

Table 2. Summary of pair-wise comparisons

		Using all 42 slides						Using 41 slides (excluding mt6CntC)					
Fold Change		-	-	1.5-fold	1.5-fold	2-fold	2-fold	-	-	1.5-fold	1.5-fold	2-fold	2-fold
P value (T-test)		0.05	0.01	0.05	0.01	0.05	0.01	0.05	0.01	0.05	0.01	0.05	0.01
A	WT 6H	8	6	8	6	8	6	8	6	8	6	8	6
B	WT 24H	489	330	489	330	434	305	581	385	581	385	496	350
C	WT 48H	1613	1235	1611	1235	1438	1155	1826	1413	1823	1413	1598	1294
D	mt 6H	0	0	0	0	0	0	0	0	0	0	0	0
E	mt 24H	0	0	0	0	0	0	0	0	0	0	0	0
F	mt 48H	0	0	0	0	0	0	0	0	0	0	0	0
G	WTvsmt @0H	0	0	0	0	0	0	0	0	0	0	0	0
H	WTvsmt @6H	0	0	0	0	0	0	0	0	0	0	0	0
I	WTvsmt @24H	0	0	0	0	0	0	0	0	0	0	0	0
J	WTvsmt @48H	0	0	0	0	0	0	0	0	0	0	0	0

Fold Change: Cut-off value of minimum fold change. “-“ indicates no filtering by fold change.

P value (T-test): Cut-off value of maximum P value calculated by AffylmGUI. The value is calculated by Student's T-test and adjusted for FDR.

A - C: difference between imazapyr-treated wildtype and mock-treated wildtype at 6, 24 and 48 hours of treatment.

D - F: difference between imazapyr-treated *csr1-2*^{D} and mock-treated *csr1-2*^{D} at 6, 24 and 48 hours of treatment.

G - J: difference between mock-treated *csr1-2*^{D} and mock-treated wildtype at 0, 6, 24 and 48 hours of treatment.

Table 3. Pearson's correlation coefficient of co-expressed genes

AGI	Gene title	Pearson's correlation coefficient (r > 0.75)							
		At1g05680	At3g22370	At2g41730	At2g03760	At5g62480	At5g43450	At4g37370	At2g04040
At1g05680	UDP-glucoronosyl/UDP-glucosyl transferase family protein	1	0.899	0.928	0.939	0.879	0.928	0.857	0.822
At3g22370	alternative oxidase 1a, mitochondrial (AOX1A)	0.899	1	0.946	0.916	0.899	0.864	0.923	
At2g41730	expressed protein	0.928	0.946	1	0.974	0.949	0.833	0.977	
At2g03760	steroid sulfotransferase, putative	0.939	0.916	0.974	1	0.945	0.845	0.949	
At5g62480	glutathione *S*-transferase, putative	0.879	0.899	0.949	0.945	1		0.942	
At5g43450	2-oxoglutarate-dependent dioxygenase, putative	0.928	0.864	0.833	0.845		1		0.843
At4g37370	cytochrome P450, putative	0.857	0.923	0.977	0.949	0.942		1	
At2g04040	MATE efflux family protein	0.822					0.843		1
At3g50930	AAA-type ATPase family protein	0.793	0.865	0.94	0.92	0.917		0.968	
At1g01480	1-aminocyclopropane-1-carboxylate synthase 2	0.836	0.895	0.959	0.939	0.937		0.968	
At3g61630	AP2 domain-containing transcription factor, putative	0.818	0.886	0.944	0.927	0.943		0.966	
At2g04050	MATE efflux family protein	0.938	0.863	0.92	0.959	0.876	0.873	0.876	0.769
At1g32870	no apical meristem (NAM) family protein	0.877	0.959	0.953	0.915	0.911	0.796	0.928	
At1g76600	expressed protein	0.773	0.853	0.924	0.877	0.875		0.956	
At4g04490	protein kinase family protein	0.931	0.85	0.914	0.954	0.869	0.865	0.87	
At2g04070	MATE efflux family protein	0.949	0.832	0.862	0.888	0.826	0.922	0.774	0.901
At2g46310	AP2 domain-containing transcription factor, putative	0.84	0.853	0.948	0.924	0.924		0.946	
At2g32020	GCN5-related N-acetyltransferase (GNAT) family protein		0.854	0.914	0.862	0.904		0.947	
At1g71330	ABC transporter family protein	0.946	0.858	0.871	0.887	0.831	0.911	0.799	0.861
At1g10170	NF-X1 type zinc finger family protein	0.871	0.912	0.946	0.944	0.935	0.761	0.943	
At2g38250	DNA-binding protein-related	0.774	0.856	0.91	0.892	0.913		0.945	
At1g42990	bZIP transcription factor family protein	0.828	0.92	0.931	0.898	0.908		0.939	
At1g21520	expressed protein	0.768	0.847	0.916	0.868	0.907		0.935	
At2g44070	eukaryotic translation initiation factor 2B family protein		0.81	0.892	0.862	0.892		0.922	
At3g55620	eukaryotic translation initiation factor 6, putative	0.918	0.847	0.898	0.913	0.882	0.793	0.847	
At2g18190	AAA-type ATPase family protein	0.811	0.802	0.895	0.916	0.88		0.887	
At2g20720	pentatricopeptide (PPR) repeat-containing protein	0.915	0.821	0.855	0.876	0.762	0.861	0.792	
At2g47000	multidrug resistant (MDR) ABC transporter, putative		0.766	0.838	0.816	0.914		0.862	
At2g30250	WRKY family transcription factor	0.783	0.828	0.896	0.909	0.892		0.913	
At2g35480	expressed protein	0.772					0.778		0.912
At2g47520	AP2 domain-containing transcription factor, putative	0.782	0.823	0.886	0.856	0.889		0.902	
At2g20800	pyridine nucleotide-disulphide oxidoreductase family protein	0.793	0.768	0.863	0.898	0.828		0.854	
At3g26830	cytochrome P450 71B15, putative (CYP71B15)	0.809					0.797		0.896
At5g59820	zinc finger (C2H2 type) family protein (ZAT12)		0.787	0.857	0.825	0.853		0.895	
At5g40690	expressed protein			0.799	0.836	0.893		0.82	
At5g54100	band 7 family protein	0.892		0.771	0.812	0.762	0.831		0.88
At1g05060	expressed protein		0.78	0.855	0.891	0.883		0.888	
At2g32930	zinc finger (CCCH-type) family protein		0.784	0.863	0.883	0.851		0.886	
At1g31290	PAZ domain-containing protein		0.773	0.854	0.802	0.815		0.872	
At5g59450	scarecrow-like transcription factor 11 (SCL11)		0.831	0.848	0.773	0.828		0.869	
At3g28580	AAA-type ATPase family protein	0.755	0.809	0.844	0.81	0.849		0.867	
At2g21640	expressed protein	0.815					0.853		0.862
At5g63790	no apical meristem (NAM) family protein			0.789	0.756	0.808		0.859	
At1g16670	protein kinase family protein			0.79		0.828		0.85	
At3g11020	DRE-binding protein (DREB2B)		0.775	0.827	0.848	0.793		0.842	
At5g25260	expressed protein	0.822			0.769		0.761		0.848
At1g24090	RNase H domain-containing protein								0.837
At2g18180	SEC14 cytosolic factor, putative			0.822	0.836	0.809		0.831	
At4g08850	leucine-rich repeat family protein			0.81	0.821	0.809		0.835	
At3g22910	Ca(2+)-ATPase, putative (ACA13)		0.774	0.799		0.777		0.834	
At4g05020	NADH dehydrogenase-related		0.786	0.759	0.753	0.83		0.794	
At4g21680	proton-dependent oligopeptide transport (POT) family protein			0.773	0.778	0.758		0.829	
At1g43910	AAA-type ATPase family protein			0.77	0.769	0.817		0.757	
At1g15430	expressed protein			0.773		0.798		0.816	
At1g17180	glutathione *S*-transferase, putative					0.814		0.775	
At1g23550	expressed protein	0.779					0.788		0.805
At1g07520	scarecrow transcription factor family protein					0.801			
At2g20950	expressed protein					0.752		0.799	
At1g62300	WRKY family transcription factor					0.787		0.796	
At1g34180	no apical meristem (NAM) family protein					0.795			
At1g35230	arabinogalactan-protein (AGP5)	0.793					0.789		0.783
At1g20350	mitochondrial import inner membrane translocase subunit Tim17, putative						0.785		0.79
At1g55920	serine O-acetyltransferase, putative					0.79		0.776	
At5g56350	pyruvate kinase, putative					0.788			
At2g22010	zinc finger (C3HC4-type RING finger) family protein					0.783		0.764	
At1g01720	no apical meristem (NAM) family protein					0.751		0.779	
At1g61640	ABC1 family protein								0.777
At3g19580	zinc finger (C2H2 type) protein 2 (AZF2)							0.776	
At2g36790	UDP-glucoronosyl/UDP-glucosyl transferase family protein						0.776		
At4g18880	heat shock transcription factor 21 (HSF21)					0.762		0.764	
At4g01870	tolB protein-related							0.762	
At4g22590	trehalose-6-phosphate phosphatase, putative							0.761	
At1g26420	FAD-binding domain-containing protein		0.76						
At5g62490	ABA-responsive protein (HVA22b)					0.758			
At3g04240	O-linked N-acetyl glucosamine transferase, putative					0.758			

List of Pearson's correlation coefficient (r) values for eight genes up-regulated in imazapyr-treated wildtype shoots after six hours of treatment (top eight genes written in bold) along with genes which have high r values (r > 0.75) with at least one of these eight genes. r values less than 0.75 were omitted.

Table 4. Frequency of imazapyr regulation within detoxification related gene families

Family	Total	ATH1	Regulated	Percentage	Data Source
Glycosyltransferase	361	262	10	3.82	TAIR
GSTs	53	50	9	18.00	TAIR
Cytochrome P450	256	209	8	3.83	TAIR
ABC Superfamily	129	114	10	8.77	TAIR
ABC Superfamily	117	107	10	9.35	PlantsT
ABC transporters	94	85	8	9.41	TAIR
MATE family protein	56	52	8	15.38	PlantsT
Alternative Respiratory	14	14	5	35.71	Clifton et al. 2005
ST	18	16	1	6.25	Klein and Papenbrock, 2004
ACO	9	8	1	12.50	De Paepe et al. 2004/TAIR

Family: Name of family

Total: Number of family members

ATH1: Number of genes represented in ATH1 GeneChip®

Regulated: Number of genes which are regulated by imazapyr treatment in wildtype at at least one of time points.

Percentage: Percentage of [Regulated] per [ATH1]

Data Source: Source for gene IDs for family member genes.

Table 5. List of imazapyr-regulated genes belonging to families of glycosyltransferase, GST superfamily, cytochrome P450, ABC superfamily, MATE, ST and ACO and components of alternative respiratory pathway.

	AGI	Probe Set ID	Gene Name	Family	6 Hour		24 Hour		48 Hour	
					M	P	M	P	M	P
Glycosyltransferase										
	At1g05560	263184_at	UGT75B2	Family 1					2.12	0.003
	At1g05680	263231_at		Family 1	3.78	0.003	6.39	0.000	6.89	0.000
	At2g15490	265501_at		Family 1			1.85	0.000	2.06	0.000
	At2g30140	267300_at		Family 1			2.45	0.001	3.06	0.000
	At2g36750	265197_at		Family 1			1.67	0.003	2.39	0.000
	At2g36790	265200_s_at		Family 1			3.42	0.000	5.18	0.000
	At2g36970	263847_at		Family 1					1.72	0.008
	At4g02280	255521_at		Family 4			1.26	0.036	2.32	0.000
	At1g53290	260644_at		Family 31					-1.56	0.000
	At3g04240	258575_at		Family 41					1.20	0.000
GST superfamily										
	At5g16710	246454_at	AtDHAR3	Dehydroascorbate reductase family					-1.30	0.000
	At1g10360	264435_at	AtGSTU18	Tau family					-1.79	0.000
	At1g17170	262518_at	AtGSTU24	Tau family			4.54	0.000	4.66	0.000
	At1g17180	262517_at	AtGSTU25	Tau family			4.05	0.000	4.07	0.000
	At1g78370	260745_at	AtGSTU20	Tau family					-1.29	0.005
	At2g29460	266267_at	AtGSTU4	Tau family					1.89	0.003
	At2g29490	266290_at	AtGSTU1	Tau family					1.93	0.022
	At5g62480	247435_at	AtGSTU9	Tau family	2.82	0.034	5.54	0.000	5.64	0.000
	At2g02390	266181_at	AtGSTZ1	Zeta family			1.57	0.005	1.54	0.006
Cytochrome P450										
	At2g34500	266995_at	CYP710A1	CYP710A			2.92	0.001	3.10	0.000
	At3g26200	257636_at	CYP71B22	CYP71B					-1.54	0.013
	At3g26220	257624_at	CYP71B3	CYP71B					1.52	0.003
	At3g26830	258277_at	CYP71B15	CYP71B			3.52	0.046		
	At1g11600	262819_at	CYP77B1	CYP77B					-1.06	0.026
	At4g37370	253046_at	CYP81D8	CYP81D	2.99	0.028	4.60	0.000	4.91	0.000
	At1g64900	262882_at	CYP89A2	CYP89A			2.54	0.000	2.30	0.000
	At3g03470	259058_at	CYP89A9	CYP89A					2.02	0.020
ABC Superfamily										
	At2g47000	266752_at	MDR4	3.A.1.201.1 MDR			5.41	0.000	5.93	0.000
	At3g28860	257137_at	MDR11	3.A.1.201.1 MDR					-1.52	0.000
	At2g34660	267319_at	MRP2	3.A.1.208.1 CT2					1.61	0.001
	At2g47800	266464_at	MRP4	3.A.1.208.1 CT2			1.96	0.000	2.52	0.000
	At3g13090	257184_at	MRP8	3.A.1.208.1 CT2					1.27	0.000
	At3g60160	251457_s_at	MRP9	3.A.1.208.1 CT2					1.48	0.008
	At3g59140	251503_at	MRP14	3.A.1.208.1 CT2			1.63	0.000	2.21	0.000
	At4g19210	254615_at	RLI2	Unknown Family					1.29	0.000
	At5g60790	247593_at	GCN1	GCN subfamily					1.59	0.001
	At1g71330	259937_s_at	NAP5	Unknown Family			4.82	0.000	5.67	0.000
MATE										
	At1g33110	261618_at					1.55	0.008	1.77	0.001
	At1g71140	259743_at							1.61	0.000
	At2g04040	263403_at			2.90	0.002	5.19	0.000	5.59	0.000
	At2g04050	263402_at					6.52	0.000	7.28	0.000
	At2g04070	263401_at					4.85	0.000	5.55	0.000
	At3g21690	258179_at							1.96	0.000
	At4g25640	254077_at							1.08	0.049
	At5g65380	247193_at							1.32	0.000
Alternative Respiratory										
	At3g22370	258452_at	AOX1a	Alternative oxidase	2.67	0.003	4.10	0.000	4.89	0.000
	At2g29990	266835_at	NDA2	Alternative NADH-dehydrogenase			1.42	0.044		
	At4g05020	255259_at	NDB2	Alternative NADH-dehydrogenase			3.49	0.000	3.79	0.000
	At2g20800	265422_at	NDB4	Alternative NADH-dehydrogenase			4.11	0.000	4.77	0.000
	At3g54110	251902_at	UCP1	Uncoupling protein					1.11	0.008
ST										
	At2g03760	264042_at	AtSOT12	V	2.88	0.000	4.33	0.000	4.30	0.000
ACO										
	At5g43450	249125_at			2.60	0.000	3.05	0.000	3.15	0.000

List of up- or down-regulated genes classified by families. Data sources are same as Table 3. M is log2 fold-change and P is FDR-adjusted P-value for student's T-test. Cut off is $|M| > 1$ (More than 2-fold) and $P < 0.05$.

Table 6. Expression frequency of groups of genes which are predicted to be altered in response to imazapyr treatment

Process	Total	ATH1	Regulated	Percentage	Data Source
Branched-Chain Amino Acid Biosynthesis	**34**	**29**	**1**	**3.45**	**Aracyc**
Amino Acid Biosynthesis	**205**	**177**	**17**	**9.60**	**Aracyc**
Aminoacyl-tRNA synthetase	**57**	**46**	**9**	**19.57**	**Aracyc**
Aminoacyl-tRNA synthetase	**53**	**48**	**10**	**20.83**	**Duchene et al. 2005**
Ribosomal Protein	**358**	**312**	**80**	**25.64**	**GO (ribosome)**
Secondary Metabolites Biosynthesis	**141**	**130**	**14**	**10.77**	**Aracyc**
IAA	18	14	2	14.29	
Lignin	41	40	7	17.50	
Phenylpropanoid	12	12	1	8.33	
Flavonoid	27	27	2	7.41	
Anthocyanin	18	16	0	0.00	
Flavonol	24	20	1	5.00	
Camalexin	1	1	1	100.00	
Senescence	**118**	**106**	**8**	**7.55**	**Gepstein et al. 2003**
Amino acid transport	5	4	1	25.00	
Cell wall	8	7	1	14.29	
Detoxification	13	12	0	0.00	
Lipid metabolism	3	3	0	0.00	
LSG clones with unknown functions	28	24	0	0.00	
Macromolecular degradation and recycling	11	10	0	0.00	
Metabolism	5	5	0	0.00	
Regulatory genes	18	16	5	31.25	
SSH clones involved in various processes	9	9	0	0.00	
Stress, pathogenicity and secondary metabolites	17	15	1	6.67	
Sugar metabolism	1	1	0	0.00	
Amino Acid Transporter	**60**	**56**	**1**	**1.79**	**PlantsT**
The Amino Acid-Polyamine-Organocation (APC)	14	14	0	0.00	
The Amino Acid/Auxin Permease (AAAP)	46	42	1	2.38	
Peptide Transporter	**62**	**60**	**1**	**1.67**	**PlantsT**
The Proton-dependent Oligopeptide Transporter	52	50	1	2.00	
The Oligopeptide Transporter	9	9	0	0.00	
The Salt or Low Temperature Stress-Induced Hydrophobic Peptide	1	1	0	0.00	
Senescence	**23**	**21**	**5**	**23.81**	**van der Graaff et al. 2006**
Senescence	**12**	**11**	**0**	**0.00**	**GO (leaf senescence)**
Death	**218**	**190**	**5**	**2.63**	**GO (death)**
Aging	**61**	**59**	**4**	**6.78**	**GO (aging)**

Total: Genes involved in the biological processes
Regulated: Number of genes which are regulated by imazapyr treatment in wildtype at at least one of time points.
ATH1: Number of genes represented in ATH1 Genechip®
Percentage: Percentage of [Regulated] per [ATH1]
Data Source: Source for gene IDs for genes involved in the process

Table 7. List of imazapyr-regulated genes belonging to pathways of amino acids biosynthesis and aminoacyl-tRNA synthesis.

				24 Hour		48 Hour	
AGI	Probe Set ID	Enzymatic activity	AA	M	P	M	P
Nonpolar Amino Acids (hydrophobic)							
AT1G17290	260847_s_at	alanine transaminase	Ala			1.58	0.007
At2g05710	263348_at	3-isopropylmalate dehydratase	Leu	1.18	0.000	1.59	0.000
AT3G07630	259254_at	prephenate dehydratase	Phe			1.51	0.000
AT1G29410	259770_s_at	phosphoribosylanthranilate isomerase	Trp			1.20	0.004
AT1G63660	260294_at	anthranilate synthase	Trp			1.23	0.000
AT3G27740	258243_at	anthranilate synthase	Trp	1.12	0.000	1.72	0.000
Polar Amino Acids (hydrophilic)							
AT1G17745	259403_at	phosphoglycerate dehydrogenase	Ser	2.41	0.004	3.23	0.000
AT1G55920	260602_at	serine acetyltransferase	Cys	1.35	0.000	1.26	0.002
AT3G10050	258884_at	cysteine synthase	Cys	1.13	0.031	1.91	0.000
AT3G17820	258160_at	glutamine synthetase	Gln			1.51	0.002
Electrically Charged Amino Acids (positive and hydrophilic)							
AT3G14390	258365_s_at	diaminopimelate decarboxylase	Lys			1.21	0.003
AT3G60880	251392_at	dihydrodipicolinate synthase	Lys	1.03	0.002	1.04	0.002
AT5G13280	250291_at	aspartate kinase	Lys			1.23	0.001
AT5G46180	248879_at	succinyldiaminopimelate aminotransferase	Lys			2.47	0.000
AT5G52100	248402_at	dihydrodipicolinate reductase	Lys			-1.81	0.001
AT3G20330	257680_at	ornithine carbamoyltransferase	Arg	1.12	0.009	1.28	0.001
AT1G31860	246265_at	phosphoribosyl-AMP cyclohydrolase	His			1.15	0.008
Aminoacyl-tRNA synthetase							
At1g50200	262468_at	alanine-tRNA ligase	Ala			1.13	0.017
At1g09620	264705_at	isoleucine-tRNA ligase//leucine-tRNA ligase	Leu			1.38	0.000
At1g72550	259920_at	phenylalanine-tRNA ligase	Phe			1.04	0.020
At5g38830	249537_at	cysteine-tRNA ligase	Cys			1.99	0.000
At1g25350	245631_at	glutamine-tRNA ligase	Gln			1.30	0.000
At3g11710	259069_at	lysine-tRNA ligase	Lys	1.09	0.016	1.56	0.000
At1g17960	255893_at	threonine-tRNA ligase	Thr	4.82	0.000	5.66	0.000
At4g31180	253561_at	asparagine-tRNA ligase	Asp	1.28	0.014	1.79	0.000
At1g14610	260780_at	isoleucine-tRNA ligase//leucine-tRNA ligase	Val	1.18	0.019	1.46	0.000
At2g25840	266648_at		Trp	-1.03	0.001	-1.59	0.000

List of up- or down-regulated genes classified by pathways which they are belonged to. Data sources are same as Table 5. M is log2 fold-change and P is FDR-adjusted P-value for student's T-test. Cut off is $|M| > 1$ (More than 2-fold) and $P < 0.05$.

Legends To Figures

Figure 1. *csr1-2*^{D} is tolerant to up to 1000 μg L^{-1} of imazapyr while the shoot growth of wildtype is inhibited by 10 - 1000 μg L^{-1} imazapyr.

Shoot growth reduction of wildtype (solid line) and *csr1-2*^{D} (dashed line) under imazapyr. Seven-day-old seedlings were transferred to medium supplemented with 0 to 1000 μg L^{-1} imazapyr and were allowed to grow for 14 additional days. After the treatment, excised shoots were weighed (N = 15). Error bars indicate standard errors.

Figure 2. Growth inhibition by imazapyr occurs within several hours in root and within several days in shoot.

(A): Root growth reduction of wildtype and *csr1-2*^{D} under imazapyr. Four-day-old seedlings were transferred to medium supplemented with 0 or 100 μg L^{-1} imazapyr and were allowed to grow for up to 60 additional hours. After the treatment, length of primary root growth was measured (N = 16). Error bars indicate standard errors.

(B): Shoot growth reduction of wildtype and *csr1-2*^{D} under imazapyr. Seven-day-old seedlings with four leaves were transferred to medium supplemented with 0 (control) or 100 μg L^{-1} imazapyr and were allowed to grow for up to 6 additional days. After the treatment, the number of leaves was counted (N = 15). Error bars indicate standard errors.

Figure 3. Euler diagram of imazapyr regulated genes in wildtype plants.

Each circle represents genes up- or down-regulated at certain time points (More than 2-fold change at P = 0.05; Corresponding to second right column of Table 2). Numbers in parentheses under hour indicate sums of genes up- or down-regulated at cirtain time points, and number inside each area indicates the number of genes up- or down-regulated overlappedly in two or three time points or specifically in certain time point.

Figure 4. RT-PCR of eight imazapyr-induced genes in response to various herbicides which target AHAS

Figure 5. Events after imazapyr treatment in chronological order.

Pale yellow arrow indicates events in root and Pale green arrow indicates events in shoot. Inhibition of root growth and induction of detoxification are earliest events. Then alteration of amino acids biosynthesis is observed. Alteration in biosynthesis of secondary metabolites derived from amino acids, and induction of tRNA biosynthesis are presumably the results of alteration in amino acids biosynthesis. Inhibition of shoot occurs later than the transcriptional regulation observed by microarray analysis.

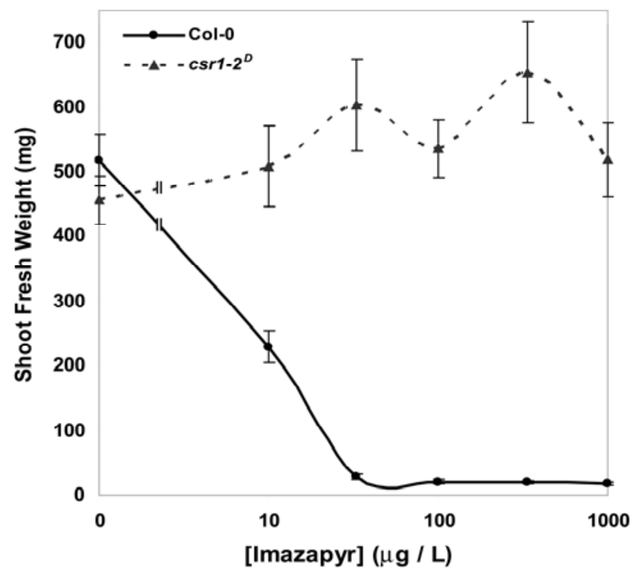

Fig. 1

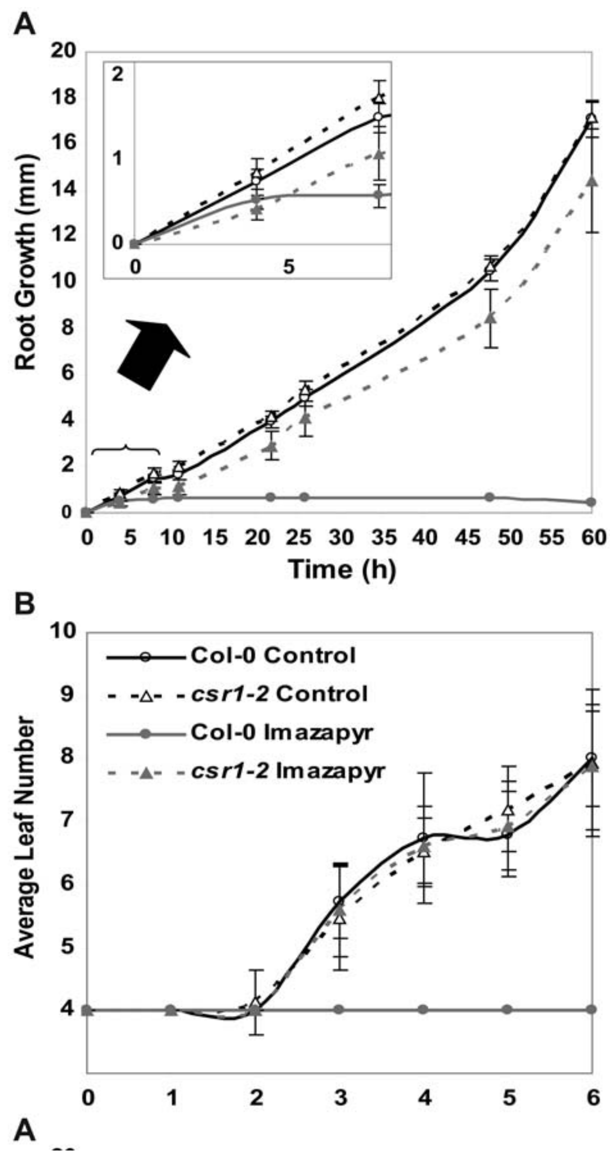

Fig. 2

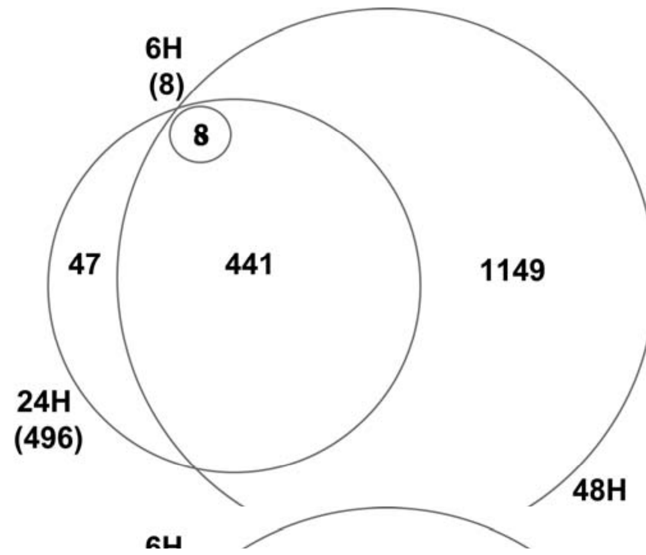

Fig. 3

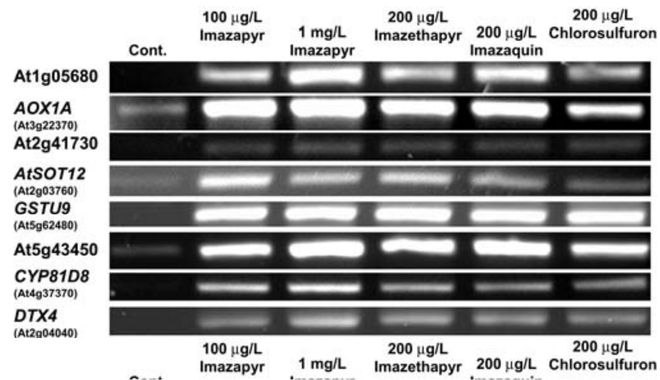

Fig. 4

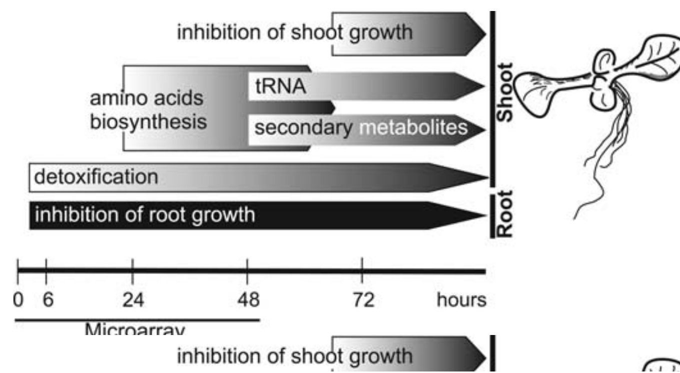

Fig. 5