Down-regulation of acetolactate synthase compromises Oi-1-mediated resistance to powdery mildew in tomato

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Abstract

Background: In a cDNA-AFLP analysis comparing transcript levels between powdery mildew (Oidium neolycopersici)-susceptible tomato cultivar Moneymaker (MM) and near isogenic lines (NILs) carrying resistance gene Oi-1 or Oi-4, a transcript-derived fragment (TDF) M11E69-195 was found to be present in NIL-Oi-1 but absent in MM and NIL-Oi-4. This TDF shows homology to acetolactate synthase (ALS). ALS is a key enzyme in the biosynthesis of branched-chain amino acids valine, leucine and isoleucine, and it is also a target of commercial herbicides.

Results: Three ALS homologs ALS1, ALS2, ALS3 were identified in the tomato genome sequence. ALS1 and ALS2 show high similarity, whereas ALS3 is more divergent. Transient silencing of both ALS1 and ALS2 in NIL-Oi-1 by virus-induced gene silencing (VIGS) resulted in chlorotic leaf areas that showed increased susceptibility to O. neolycopersici (On). VIGS results were confirmed by stable transformation of NIL-Oi-1 using an RNAi construct targeting both ALS1 and ALS2. In contrast, silencing of the three ALS genes individually by RNAi constructs did not compromise the resistance of NIL-Oi-1. Application of the herbicide chlorsulfuron to NIL-Oi-1 mimicked the VIGS phenotype and caused loss of its resistance to On. Susceptible MM and On-resistant line NIL-Oi-4 carrying a nucleotide binding site and leucine rich repeat (NB-LRR) resistance gene were also treated with chlorsulfuron. Neither the susceptibility of MM nor the resistance of NIL-Oi-4 was affected.

Conclusions: ALS is neither involved in basal defense, nor in resistance conferred by NB-LRR type resistance genes. Instead, it is specifically involved in Oi-1-mediated resistance to tomato powdery mildew, suggesting that ALS-induced change in amino acid homeostasis is important for resistance conferred by Oi-1.

Keywords: Acetolactate synthase, Oidium neolycopersici, Resistance, Solanum lycopersicum, Amino acid homeostasis

Background

In their natural environment plants are constantly attacked by a variety of pathogens. Nevertheless, plants can detect and evade most infection attempts through constitutive and inducible immune responses. The inducible responses consist of two layers [1]. The first layer is triggered by multifarious pathogen-associated molecular patterns (PAMPs). The perception of PAMPs by plant pattern recognition receptors (PRRs) stimulates a number of cellular events, which include production of reactive oxygen species, activation of mitogen-activated kinases, enhanced expression of defense genes and production of antimicrobial compounds [2,3]. The second layer of inducible responses is activated by variable pathogen-specific effectors. Recognition of effectors by the plant is mostly mediated by a class of resistance proteins which contain nucleotide binding site and leucine rich repeat (NB-LRR) domains. The regulation and execution of both inducible responses involve hormone signalling pathways [4].

Emerging evidence illustrates that defense pathways are not only regulated by classical hormones, but also amino acid metabolic pathways constitute an important part of the plant immune system [5]. Besides the fact...
that some amino acids serve as precursors of antimicrobial compounds (e.g. glucosinolates) [6], amino acid homeostasis is pivotal for the outcome of plant-microbe interactions. A dominant nematode resistance gene in soybean encodes a serine hydroxymethyltransferase (SHMT), which plays a key role in one-carbon folate metabolism [7]. The SHMT allele in the resistant genotype encodes an isoform of the enzyme with altered kinetic properties compared with the isoform in susceptible genotypes. This altered SHMT enzyme is likely associated with perturbation of the folate pathway resulting in nutritional deficiency for nematodes. Overexpression of a pepper arsenic synthetase in Arabidopsis enhanced the resistance to bacterial and oomycete pathogens, which was correlated with increased arsenic levels [8]. Arabidopsis recessive downy mildew-resistant (dmr1) mutants defective in homoserine kinase were found to be resistant to the oomycete Hyaloperonospora arabidopsidis (Hpa). The resistance was homoserine-induced, and independent of known signalling pathways [9]. Suppression of the ortholog SIDM1 in tomato resulted in elevated resistance to powdery mildew Oidium neolycopersici [10]. Resistance to Hpa was also obtained in Arabidopsis rarr1-suppressor (rsp) mutants, in which the level of threonine (Thr) was highly elevated [11]. The rsp1 mutant carries a mutation in the aspartate kinase2 gene [11], which catalyzes the first step in the aspartate-derived amino acid pathway. The rsp2 mutant contains a loss-of-function allele of dihydrodipicolinate synthase2 [11], which is the key enzyme in lysine biosynthesis. Disruption of an amino acid transporter LHT1 confers a broad spectrum disease resistance in Arabidopsis plants, likely as a result of deficiency of glutamine [12].

Oidium neolycopersici (On) is an important biotrophic fungal disease for greenhouse crops. Unlike most powdery mildews that are host specific, On can infect a wide range of hosts, including species of the Solanaceae and Cucurbitaceae families [13]. A favourable strategy to control the disease consists of exploration of resistant alleles from wild species and introgression of these alleles into cultivated species to develop resistant cultivars. In tomato nine loci conferring resistance to On have been identified [14,15]. One of them - Ol-1 - originates from Solanum habrochaites G1.1560 [16], and confers incomplete resistance associated with slow hypersensitive response (HR) [17]. It is located on chromosome 6 [16,18] and has been fine-mapped to a region encompassing six predicted genes, based on the sequence of tomato cultivar Heinz 1706 [19] & unpublished results. None of the six genes encodes a protein with NB-LRR domains. Unravelling the identity of Ol-1 has not been successful yet, because silencing of the predicted candidate genes individually did not attenuate the resistance level of the near-isogenic line carrying Ol-1 (NIL-Ol-1) [unpublished results]. Another resistance gene - Ol-4 - which has been introgressed from S. peruvianum LA2172 confers complete resistance to On with fast HR [16]. It has been mapped to the Mi-1 gene cluster on chromosome 6 [15]. Disease tests showed that NIL-Ol-4 was resistant to root-knot nematodes, indicating the presence of a functional Mi-1 homolog encoding a NB-LRR type protein. Furthermore, silencing of Mi-1 homologs in NIL-Ol-4 compromised the resistance to both On and root-knot nematodes, showing that Ol-4 is a Mi-1 homolog [20].

In a previous study designed to elucidate the pathways of On resistance, a cDNA-AFLP approach was used to identify transcript-derived fragments (TDF) showing differential presence or intensity in resistant tomato NILs relative to susceptible Moneymaker (MM) after mock-inoculation or inoculation with powdery mildew On [17,21]. A BLAST analysis of the sequences of a number of differentially expressed TDFs was performed using the Sol Genomics Network (SGN) database to identify unigene sequences showing highest homology to each TDF. Subsequently, Tobacco Rattle Virus (TRV)-based Virus-Induced Gene Silencing (VIGS) constructs were generated targeting the unigenes. Then, VIGS was performed in the genotypes in which the TDF was detected to analyse whether silencing of targeted genes altered On resistance. In this way, it was shown that a putative glutathione S-transferase gene is required for Ol-1-mediated resistance against On [22].

In the present study we focused on another of these differentially expressed TDF (M11E69-195) and analysed its involvement in On resistance. M11E69-195 was specifically present in NIL-Ol-1 but absent in MM and NIL-Ol-4 [17,21]. The sequence of this TDF showed homology to acetolactate synthase (ALS). ALS (EC 2.2.1.6) is more frequently referred to as acetohydroxyacid synthase (AHAS) [23] in other studies. In this study, we describe it as ALS based on the annotation in the SGN database. ALS catalyzes the first step in the production of the branched-chain amino acids (BCAAs) valine, leucine and isoleucine [24]. It is extensively studied since it is a target of commercially successful herbicides. Different herbicide molecules can block substrate access to the active site of the ALS enzyme [25]. Here, we report the involvement of ALS in Ol-1-mediated resistance to powdery mildew in tomato.

Results
Down-regulation of two ALS genes simultaneously compromises Ol-1-mediated resistance to powdery mildew On
In the cDNA-AFLP study by Li et al. [17,21] TDF fragment M11E69-195 (No. 24 in Appendix 1 in [17]; No. 71 in Appendix 1 in [21]) was observed to be present inOn-resistant NIL-Ol-1, but absent in On-susceptible MM and On-resistant NIL-Ol-4. BLAST analysis of the
sequence of this 195-bp TDF fragment was initially performed using the SGN database before the tomato genome sequence became publicly available. Highest homology was obtained for unigene SGN-U196237, a *Capsicum annuum* ALS gene (Additional file 1A). Primers were designed based on the U196237 sequence, and a 287-bp PCR product obtained using NIL-Ol-1 cDNA as template was cloned into VIGS vector TRV2, resulting in vector TRV-U196237 (Figure 1). Infiltration of TRV-U196237 into NIL-Ol-1 induced morphological changes, including short stature and curled leaves with chlorotic areas (Figure 2A). Subsequently, VIGS plants were inoculated with *On*. Quantification of fungal biomass showed that there was a significant (3-fold) increase on plants infiltrated with TRV-U196237 compared to plants infiltrated with the empty vector (TRV-EV) (Figure 2B).

After the tomato genome sequence became accessible a new BLAST analysis of the sequence present in the VIGS vector was performed. This resulted in the identification of three putative ALS genes in tomato named ALS1 (*Solyc03g044330*), ALS2 (*Solyc07g061940*) and ALS3 (*Solyc06g059880*) (Additional file 1B). The latter one, although present on chromosome 6, does not reside in the Ol-1 region [19]. ALS1 and ALS2 predicted proteins are 94% identical at the amino acid level, while ALS3 is quite different from ALS1 and ALS2 (75% and 78% identity with ALS1 and ALS2, respectively) (Additional file 1C). The ALS1 and ALS2 genes are predicted to contain one exon, whereas ALS3 is predicted to contain three exons (Figure 1). Alignment of the TDF sequence (derived from the NIL-Ol-1 line) with the three annotated ALS genes showed that the TDF was probably derived from the ALS3 ortholog in *S. habrochaites* (Additional file 1A). However, alignment of the cloned fragment in the VIGS construct with the three annotated ALS genes resulted in highest homology to ALS2. This discrepancy can be explained, because for construction of the VIGS vector primers were designed based on the SGN unigene showing highest homology to the TDF, but no unigene based on EST sequences from ALS3 was present in the SGN database. The alignment suggested that the VIGS vector targeted both ALS1 and ALS2, but not ALS3, based on the assumption that an identical sequence of at least 21 nucleotides is necessary for efficient silencing. To validate the specificity of silencing, transcript levels of ALS1, ALS2 and ALS3 in NIL-Ol-1 plants subjected to VIGS were measured by qRT-PCR using RNA isolated after pooling the third and fourth whole leaves of each plant. In this experimental set-up expression levels of the three ALS genes were not significantly reduced in TRV-U196237-infiltrated plants compared with TRV-EV-
infiltrated plants (data not shown), although the alteration in leaf morphology indicated a VIGS effect. However, we noticed that fungal colony growth was stronger on the chlorotic areas of the leaves than on the green areas. Therefore, transcript levels of ALS1, ALS2, and ALS3 were compared between leaf samples collected from excised green and chlorotic areas of TRV-U196237-infiltred plants. The expression levels of ALS1 and ALS2 were significantly lower in chlorotic areas in comparison with green areas, while expression of ALS3 was also somewhat decreased in chlorotic areas, but not significantly (Figure 2C). This indicated that the fragment present in the TRV-U196237 VIGS construct specifically silenced ALS1 and ALS2, but not ALS3.

To confirm the results obtained with VIGS, we generated stable transformants of NIL-Ol-1 in which both ALS1 and ALS2 were silenced by an RNAi construct (RNAi-ALS1 + 2) containing an inverted repeat of the same sequence as present in the TRV-U196237 vector. We expected that when ALS1 and ALS2 were efficiently silenced by RNAi the transformants would show the same visible phenotype as in VIGS, i.e. smaller plants with chlorotic leaves. Nine primary transformants (T1) were selfed to produce T2 families. For each T2 family ten plants were phenotypically examined. One T2 family (216) showed clear segregation for the phenotypic traits mentioned above (reduced stature and chlorotic leaves) (Figure 3A). The altered phenotype co-segregated with the presence of the silencing construct, as indicated by amplification of the 35S promoter (Figure 3A). After inoculation with On the T2 plants with an altered phenotype showed increased sporulation when compared with the untransformed NIL-Ol-1 plants (Figure 3B), although full susceptibility as in cultivar MM was not reached. Fungal growth and transcript levels of ALS genes were quantified in NIL-Ol-1 plants and in T2 plants with altered phenotype. The results showed that the On fungal biomass in these T2 plants was significantly increased compared with NIL-Ol-1 (Figure 3C). As expected, the gene expression levels of both ALS1 and ALS2 were significantly reduced in the RNAi-ALS1 + 2 T2 plants, whereas ALS3 expression was not significantly reduced (Figure 3D).

In addition to the production of stable transformants in which both ALS1 and ALS2 were silenced simultaneously, stable NIL-Ol-1 transformants were produced in which the three ALS genes were silenced individually to evaluate their involvement in Ol-1 resistance. No cross-silencing was observed (Additional file 2). Transformed T1 plants were selled to obtain T2 families. One T2 family for ALS1, three for ALS2, and two for ALS3 were obtained. The NPTII-containing, and thus transgenic, T2
plants were selected by PCR analysis. The transgenic T2 progeny showed a significant reduction of expression of the targeted ALS gene (Figure 3F). Silencing of the three ALS genes individually did not lead to morphological alteration, and fungal abundance was not enhanced compared to that in untransformed NIL-Ol-1 plants (Figure 3E). The fact that suppression of individual ALS genes did not compromise Ol-1-mediated resistance, but suppression of at least two ALS genes compromised the resistance indicated that the function of ALS genes is likely overlapping.

We did not try to generate a construct targeting all three genes simultaneously, because no continuous stretch of at least 21 identical nucleotides is present when aligning the complete coding sequences of the three ALS genes (Additional file 1B).

ALS is specifically involved in Ol-1-mediated resistance

Acetolactate synthase is a well-known target for commercial herbicides, which block binding of substrates to the active site of the ALS enzyme [25]. We employed this system to determine whether ALS was generally involved in powdery mildew resistance or specifically involved in Ol-1-mediated resistance. The herbicide chlorsulfuron was used as the ALS inhibitor. First, we studied the effect of herbicide treatment in NIL-Ol-1

Figure 3 Assessment of resistance to Oidium neolycopersici (On) in RNAi transforms of NIL-Ol-1. (A) Segregation for phenotypic traits in T2 plants obtained after selfing RNAi-ALS1 + 2 transformant 216, compared with the NIL-Ol-1 untransformed plant. PCR analysis of the 35S promoter of the silencing construct showed co-segregation of altered phenotype with presence of the expected PCR product. (B) On symptoms on MM, NIL-Ol-1 and NIL-Ol-1 transformant obtained with the ALS1 + 2 RNAi construct (T2 plant). (C), On fungal biomass and (D), transcript levels of ALS1, ALS2, and ALS3 in RNAi-ALS1 + 2 T2 plants of family 216 showing an altered phenotype, compared with that in NIL-Ol-1 plants. (E), On fungal biomass and (F), transcript levels of ALS1, ALS2, and ALS3 in NPTII-containing T2 plants in which three ALS genes were targeted individually by RNAi (RNAi-ALS1, RNAi-ALS2, and RNAi-ALS3). For each plant RNA was isolated from pooled 3rd and 4th leaves. Values were normalized relative to EF, and calibrated to the levels in untransformed NIL-Ol-1 plants. Error bars represent standard deviation of three biological replicates. Asterisks indicate significant difference from the controls according to independent-samples t-test and one way analysis of variance (P <0.05).
plants. As chlorsulfuron was dissolved in acetone, we included plants to which only acetone was applied as well as plants to which water was applied as controls. The herbicide application caused inhibition of shoot growth and overall chlorosis of the plant (Figure 4A). Quantification of fungal DNA showed that a significant increase of fungal biomass was attributable to herbicide treatment, as compared with the acetone control (Figure 4A and B).

As powdery mildew fungi depend on living tissue for nutrient uptake, we wondered whether the probable perturbation of amino acid homeostasis due to silencing of ALS could be exploited by the pathogen, and in turn influence the basal defense. To address this question we treated susceptible tomato MM with chlorsulfuron. If ALS is important for basal defence against powdery mildew, one would anticipate an increase of sporulation. After herbicide treatment we observed morphological changes in MM plants, which were similar to those in NIL-OL-1 plants. However, fungal biomass in chlorsulfuron-treated MM plants was similar to fungal biomass in water- and acetone-treated MM, suggesting that ALS was not involved in basal defence (Figure 4B). Chlorsulfuron was also applied to NIL-OL-4 plants to determine whether ALS is generally involved in powdery mildew resistance signalling pathways. Quantification of fungal biomass showed that herbicide-treated NIL-OL-4 plants retained a similar resistance level to powdery mildew as the control NIL-OL-4 plants, suggesting that ALS is dispensable for resistance conferred by OL-4, encoding a NB-LRR type protein (Figure 4B).

Similar to the results obtained with the VIGS and RNAi plants, we observed that, although NIL-OL-1 plants in which ALS function was impaired showed increased susceptibility to On, full susceptibility as in cultivar MM was not reached. This is shown in Figure 4C, in which fungal biomass in NIL-OL-1 and NIL-OL-4 plants is calibrated to the level in water-treated MM.

Expression of ALS genes upon powdery mildew attack in NIL-OL-1 and MM
To investigate the response of three ALS genes under powdery mildew attack, their transcript levels were measured in NIL-OL-1 and MM. Expression of ALS1 and ALS2 was detected in both genotypes, and they were not induced by powdery mildew inoculation in either genotype (Figure 5). ANOVA analysis indicated that there was no significant difference in expression levels of

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**Figure 4** ALS is specifically involved in OL-1-mediated resistance against *Oidium neolycopersici* (On). (A). Phenotype of NIL-OL-1 plants in soil to which acetone (+ Acetone) or chlorsulfuron dissolved in acetone (+ Chlorsulfuron) has been added, and close-up of On development on the leaves. (B). Relative On fungal biomass on NIL-OL-1, Moneymaker (MM) and NIL-OL-4 plants grown in soil to which water (H2O), acetone or chlorsulfuron has been added. For each plant DNA was extracted from pooled 3rd and 4th leaves. Values are normalized relative to EF, and calibrated to level on plants grown in soil with acetone. Error bars represent three biological replicates for H2O and acetone treatments respectively, and 5 or more replicates for chlorsulfuron treatment. Two independent experiments were performed with similar results, and data from one experiment are presented. Asterisk indicates significant difference from the controls according to one way analysis of variance (P <0.05). (C). Relative On fungal biomass on NIL-OL-1, MM and NIL-OL-4 plants as in Panel B, but calibrated to the level on water-treated MM plants.
ALS1 and ALS2 between NIL-Ol-1 and MM. ALS3 expression was only detected in NIL-Ol-1, while in MM it may be either weakly expressed below detection level, or not expressed at all (Figure 5). We could exclude the possibility that primers for quantifying ALS3 expression were not suitable for MM because a PCR product of the expected size was obtained using genomic DNA as template. Further, RNA-seq data of ALS3 (Solyc06g059880) from tomato cultivar Heinz [26] confirm that ALS3 is not expressed in tomato leaves (Additional file 3).

Discussion

In a screen of differentially expressed transcripts showing a difference in presence or intensity when comparing powdery mildew-resistant NILs with susceptible MM, TDF M11E69-195 was identified [17,21], which shows homology to acetolactate synthase. This TDF was specifically present in NIL-Ol-1, but absent in both MM and NIL-Ol-4 [17,21]. By targeting acetolactate synthases via VIGS, RNAi and herbicide application, we demonstrated that ALS activity was specifically important for Ol-1-based resistance (Figures 2B, 3C and 4B). ALS does not seem to be involved in basal defense as indicated by unchanged susceptibility after herbicide treatment of MM, nor required for resistance controlled by NB-LRR-type resistance genes as indicated by the results from herbicide treatment of NIL-Ol-4 (Figure 4B).

Possible involvement of ALS3 in Ol-1-mediated resistance to On

The fact that TDF M11E69-195 was observed in NIL-Ol-1, but absent in both MM and NIL-Ol-4 could be caused solely by the presence of nucleotide polymorphisms between MM and NIL-Ol-1. However, we have shown that the corresponding ALS3 gene is truly differentially expressed, as ALS3 transcripts were observed in leaves from NIL-Ol-1 but not in MM leaves (Figure 5).

The sequence of M11E69-195 from NIL-Ol-1 showed higher similarity to ALS3 than to ALS1 and ALS2 from S. lycopersicum (Additional file 1A). ALS3 (Solyc06g059880) is located on the long arm of chromosome 6, but not in the Ol-1 region. ALS1 and ALS2 are located on chromosomes 3 and 7, respectively. As NIL-Ol-1 only contains (part of) chromosome 6 of S. habrochaites G1.1560 whereas all other chromosomes are from S. lycopersicum MM, we expected that the ALS1 and ALS2 genes from NIL-Ol-1 were identical to those from MM. This was indeed observed after sequencing complete ALS1 and ALS2 cDNAs from NIL-Ol-1 (data not shown). In contrast, sequencing of the complete ALS3 cDNA from NIL-Ol-1 revealed a number of SNPs and indels in NIL-Ol-1 compared to the predicted sequence from tomato cultivar Heinz in the SGN database (Additional file 1B). NIL-Ol-4, containing an introgression of part of chromosome 6 from S. peruvianum accession LA2172 [19], was shown to contain ALS1 and ALS2 sequences identical to those from MM, whereas the ALS3 sequence from NIL-Ol-4 differed from both MM and NIL-Ol-1.

The VIGS and RNAi constructs targeted ALS1 and ALS2, but not ALS3 (Figures 2C and 3D). As ALS1 and ALS2 in all three genotypes are identical, but the effect of silencing is specific for NIL-Ol-1, we wonder whether ALS3 plays a role in resistance to tomato powdery mildew conferred by Ol-1. Although ALS3 is homologous to acetolactate synthase genes whose function has been proven, the exact function of the ALS3 protein is unknown. In plants ALS is a heteromultimer, consisting of catalytic and regulatory subunits [25,27,28]. All three tomato proteins ALS1, ALS2 and ALS3 are homologous to known catalytic subunits, such as the SuRA and SuRB proteins of Nicotiana tabacum [29]. In Solanaceous species from which genome sequences are available three ALS genes coding for catalytic subunits are present. In contrast, Arabidopsis only contains one ALS gene encoding the catalytic subunit, i.e. At3g48560.

In MM and NIL-Ol-4 only ALS1 and ALS2 are expressed in leaves, while ALS3 is not. Similarly, the
orthologs of ALS3 in S. pimpinellifolium and S. tuberosum are not expressed in leaves (Additional file 3). NIL-Ol-1 is exceptional, as in this genotype ALS3 is expressed in leaves, together with ALS1 and ALS2, and therefore ALS3 may be incorporated in the ALS holoenzyme. Possibly, the presence of different catalytic subunits in the ALS holoenzyme confers different functionalities or substrate specificities. Although silencing of only ALS3 in the NIL-Ol-1 background did not result in increased susceptibility to O. sativa (Figure 3E), this does not exclude the possibility that ALS3 is involved in resistance. The obtained transformants showed significant silencing of ALS3 (Figure 3F), but no complete silencing comparable with a knock-out mutation was achieved. Additional experiments are needed to elucidate the function of ALS3 in leaves of NIL-Ol-1, for example expression of the S. habrochaites ALS3 gene in MM background.

**Involvement of amino acid homeostasis caused by altered ALS activity in Ol-1-mediated resistance**

Although acetolactate synthase is a known target of several herbicides, it is unclear how herbicide-binding affects the amino acid metabolism in plants. Scheel and Casida [30] found that chlorsulfuron treatment of soybean suspension cultures caused a decrease of the valine and leucine contents, but had no effect on other amino acids. They showed that growth inhibition by chlorsulfuron was alleviated by supplying exogenous valine or leucine, or a combination of valine, leucine and isoleucine. Consistent with a reduction of BCAAs caused by an ALS-affecting herbicide, Ray [31] observed that addition of valine and isoleucine to excised pea root cultures reversed herbicide-induced growth inhibition. Growth retardation can also result from ALS feedback inhibition by individual end products. Chen et al. [28] showed that addition of valine or leucine to the growth medium inhibited root growth of Arabidopsis seedlings, whereas addition of isoleucine had no effect. When a combination of valine + isoleucine, or leucine + isoleucine was added to the medium root growth inhibition was less pronounced, suggesting isoleucine counteracted the inhibitory effect of valine and leucine [27]. Royuela et al. [32] detected an increase in the relative proportion of some amino acids other than BCAAs in chlorsulfuron-treated wheat and maize. Höffgen et al. [33] silenced ALS in potato by antisense inhibition, resulting in a decrease of ALS activity of up to 85%. Strong silencing of ALS resulted in severe growth retardation and stunting, and leaf chlorosis. Similar phenotypic alterations were obtained by treatment with an imidazolinone herbicide. Measurement of amino acids showed an accumulation of total free amino acids as well as perturbed composition in antisense and herbicide-treated plants. Unexpectedly, instead of decreased levels, elevated amounts of amino acids including valine, leucine and isoleucine were observed, especially in older sink leaves.

Recently, another example of a link between herbicide resistance, increased amino acid levels, and resistance to fungal species was reported. Patent US8383887 [34] discloses that corn plants expressing the bacterial gdhA gene (NADPH-dependent glutamate dehydrogenase) are resistant to aflatoxin accumulation following Aspergillus infection. Furthermore, corn and tobacco plants transformed with the gdhA gene are resistant to root rot following Fusarium virguliforme infection. Previously, it has been shown that tobacco plants transformed with the gdhA gene show an increased level of resistance to the herbicide glufosinate [35] and that total free amino acids were increased in these plants [36,37].

Taken together, the effect of ALS inhibition on levels of individual amino acids is difficult to predict, as it seems to depend on the level of residual ALS activity in different tissues, and the feedback-inhibition effect of (combinations) of individual amino acids. Despite this, we investigated whether BCAAs contents influence powdery mildew susceptibility or resistance in tomato cultivar MM and NIL-Ol-1 by exogenously applying leucine, isoleucine and valine [10] & Additional file 4. Homoserine and threonine were also included in the experiment, because they were found to affect plant immunity, and threonine is the precursor of isoleucine. If a higher level of BCAAs contributes to Ol-1-mediated resistance, we expected to gain powdery mildew resistance to some degree in MM with elevated BCAAs levels. Quantification of fungal DNA showed that only exogenous application of homoserine significantly reduced the susceptibility of MM and increased resistance of NIL-Ol-1 to O. sativa, whereas application of the other amino acids did not alter the responses of MM and NIL-Ol-1 to O. sativa [10] & Additional file 4. We also did not observe the growth retardation which can be caused by individual end products, possibly because the concentration was not sufficiently high to cause this. The results suggested that instead of an elevated level, a reduced level of BCAAs or changed compositions of individual amino acids may play a role in Ol-1-mediated powdery mildew resistance. Amino acid deprivation is known to activate defences in Arabidopsis. For instance, the accumulation of camalexin, a pathogen-inducible antimicrobial phytoalexin was induced by BCAAs starvation [38]. An alternative hypothesis is the involvement of an amino acid-derived signal(s) in defense signalling pathways, as suggested for Arabidopsis genes ALD1 and AGD2 encoding aminotransferases [39]. Furthermore, studies on plant hormone conjugates showed that jasmonate (JA) can conjugate BCAAs [40] and, in particular, JA-isoleucine is the main bioactive form of the hormone [41]. In addition, altered expression of an
enzyme involved in conjugation affects salicylic acid-mediated disease resistance [42].

In the case of Ol-1, perturbation of amino acid balance by silencing of ALS or herbicide treatment may impair the integrity of the signalling network, leading to the loss of resistance conferred by Ol-1. The unknown identity of Ol-1 makes it harder to understand the link between Ol-1-mediated resistance and amino acid homeostasis. Cloning of Ol-1, determination of amino acid homeostasis, and dissection of changes in hormone signalling pathways will aid in understanding the requirement of ALS activity for Ol-1-based resistance and shed light on the interaction of amino acid metabolism and plant immunity.

**Conclusion**

Tomato genome encodes three ALS genes. Silencing of each of them did not attenuate Ol-1-mediated resistance to tomato powdery mildew, while down-regulation of both ALS1 and ALS2 simultaneously or inhibition of the ALS activity resulted in the loss of Ol-1-mediated resistance to tomato powdery mildew. Further research on cloning of Ol-1 and association of amino-acid homeostasis with ALS activity may provide insight into the role of amino acid metabolism in tomato resistance to powdery mildew.

**Methods**

**Plant materials, fungal isolate and inoculation**

All the near isogenic lines (NIL) have been described previously [15]. They were obtained by crossing wild tomato species containing the resistance gene(s) with S. lycopersicum cultivar Moneymaker (MM), three backcrosses with MM, followed by two selfings (BC3S2 plants). Ol-1 was introgressed from S. habrochaites G1.1560, while Ol-4 was introgressed from S. peruviana (or S. arcanum) LA2172. Oidium neolycopersici (On) isolate Netherlands was maintained on susceptible MM plants in a growth chamber at 21/19°C (day/night). Fungal spores were washed off from heavily infected tomato leaves with tap water and diluted to a concentration of 2.5 × 10^4 spores per mL. The inoculum was evenly sprayed on 3 to 4 weeks-old plants.

**Virus-induced gene silencing (VIGS)**

VIGS was performed using the TRV-based vector system [43]. Primers for the TRV2 construct targeting SGN-1196237 were Fw-U196237-caccCAATGGGGAGATCGGTCTCA and Rv-U196237-ATCTCCCCATACCCCTCCTGT. A 290–bp fragment was amplified from cDNA of NIL-Ol-1 plants, and subsequently cloned into pENTR/D-TOPO vector (Invitrogen). After verification of the sequence the fragment was introduced into the pTRV2-attR1-attR2 vector [43] via LR recombination. The resulting TRV-U196237 vector was transformed into Agrobacterium strain GV3101. To establish VIGS, cotyledons of 10-days-old plants were agroinfiltrated with a mixture of TRV1 and TRV-U196237 (combined in a 1:1 ratio). As a control, a mixture of TRV1 and empty TRV2 vector (TRV-EV) was used. Three weeks after agroinfiltration the plants were sprayed with On inoculum. Three independent experiments were performed. For two experiments disease symptoms were visually scored 21 dpi by counting the number of fungal colonies on the leaves. For one experiment silencing level and fungal growth were quantified by qRT-PCR, using RNA from five plants for TRV-EV and 10 plants for TRV-U196237.

**Generation of stable silenced lines**

To suppress both ALS1 and ALS2 by RNAi, the same fragment as used in VIGS construct TRV-U196237 was introduced into the pHellsgate8 vector [44]. For the purpose of targeting ALS1 and ALS2 separately, primers were designed based on the 3' UTR sequences. For ALS1: Fw-ALSI-caccGCAAAAGTGTGTTGTATTGT and Rv-ALSI-AGTGACATATATAACAGTGAAGAT. For ALS2: Fw-ALS2-caccGTGTATTAAAAGTTTTCTATTGTG and Rv-ALS2-TTAGCTCATAAAATAGACGCTCTAAA. To suppress ALS3, primers were designed based on the sequences in coding region: Fw-ALS3-caccTATCTGTGAAAATCCCTCTAAACAA and Rv-ALS3-TTCTTATGAGATCGACTTGGCA. Fragments amplified with abovementioned primers were introduced into pHellsgate8 vector [40] and finally transformed into Agrobacterium strain AGL1 + virG. For generation of silenced lines the protocol described by Huibers et al. [10] was used. Primary transformants (T1) were selfed to produce T2 families. For each segregating T2 family, CaMV 35S promoter primers Xho-Fw-TGCTGACCCACAGATGGTTA and 35S2-GATAGTGGGATTGTGCGTCA [45] amplifying a 756-bp fragment, or NPTII primers Fw-NPTII-TTCCCCTCGGTATCCA and Rv-NPTII-GATTGTCTGTTGTGCCCAGT amplifying a 170-bp fragment from the pHellsgate8 T-DNA, were used to select transgenic T2 plants.

**Herbicide application**

Chlorsulfuron was purchased from Aldrich-Sigma (PS-1065), and the powder was dissolved in acetone (0.2 mg/ml). The herbicide solution was applied to 30-day-old plants of MM and NIL-Ol-1 growing in ø14 cm pots in normal potting soil. Before application, watering was suspended for two days to ensure that the solution could be absorbed completely. As controls, water and acetone were applied. The chlorsulfuron solution, acetone and water were added to the soil with a pipette (8 ml per pot). After this, the plants were challenged with powdery mildew On at the same day of herbicide application. Per genotype 15 plants
Quantitative reverse transcription PCR (qRT-PCR) and data analysis

For quantification of fungal biomass, DNA or RNA extracted from tomato leaves was used. For quantification of transcript levels, RNA was used. DNA was isolated with DNeasy plant mini kit (Qiagen). Total RNA was extracted from leaflets using the RNeasy kit (Qiagen). After removal of DNA with DNase I (Invitrogen), 1 μg total RNA was used for cDNA synthesis using SuperScript II Reverse Transcriptase kit (Invitrogen). Quantitative real-time PCR was conducted using the iQ SYBR Green (Bio-Rad). The PCR amplification consisted of an initial denaturation step of 3 min at 95°C, followed by denaturation at 15 sec at 95°C, annealing and extension for 1 min at 60°C for 39 cycles, then a final melt step from 65°C to 95°C ramp with 0.5°C increments per cycle to monitor specificity. Primers used for fungal quantification were Fw-On-CGCCAAGACCTAAACAAA and Rv-On-AGCCAAGAGATCGGTGTTG. Primers for tomato elongation factor 1α (EF) were Fw-EF-GGAACATT-GAGAGGAGGCTTAAG and Rv-EF-CAACACCCACA GCAACAGTCT [46]. For detection of relative transcript levels of the ALS genes primers were Fw-ALS1-CGCTCAA-CATAATCGTCGTG and Rv-ALS1-ACGGGAAACGAAT GTTTCAG for ALS1; Fw-ALS2-CCCTCTTTCCTCCAAAATCTACCT and Rv-ALS2-TTGAACAGTGAACGCTATG for ALS2; Fw-ALS3-TTTTGCTTGCTAGCATTGGAG and Rv-ALS3- GGAGTGCATATCAATGTGAACAA for ALS3. For the time-course experiment in which the expression of three ALS genes was monitored after inoculation with On, the same set of primers was used as for detection of relative transcript levels of each ALS gene after silencing. For analysis of the relative expression level and fungal biomass the 2^ΔΔCt method as described by Livak and Schmittgen [47] was used. Data were statistically examined using independent-samples t-test, one-way analysis of variance or two-way between groups ANOVA based on Post-hoc comparisons using Tukey’s HSD test (P <0.05). All analyses were performed using SPSS Statistics 20 following the instructions of SPSS Survival Manual 4th edition [48].

Additional files

Additional file 1: Sequence alignments. (A) Sequence alignment of TDF M1E69-195 and ALS PCR fragment used in VIGS and RNAi constructs ALS1 + 2 (both from NIL-0f-1) with the corresponding part of unigene SGN-U190237 from Capsicum annum, and of ALS1, ALS2 and ALS3 transcripts from tomato cultivar Heinz. Nucleotides identical with the ALS3 sequence are highlighted. (B) Sequence alignment of ALS1, ALS2 and ALS3 coding sequences (CDS) of tomato cultivar Heinz, and the ALS3 CDS of NIL-0f-1. Nucleotides identical with the Heinz ALS3 sequence are highlighted. (C) Sequence alignment of protein sequences from tomato cultivar Heinz ALS1, ALS2 and ALS3 with ALS3 from NIL-0f-1. Amino acids identical with the ALS3 sequence are highlighted.

Additional file 2: Absence of cross-silencing by RNAi constructs targeting individual ALS genes. Cross-silencing was not detected in three representative silenced lines in each of which a specific ALS gene was targeted by RNAi (RNAi-ALS1, 2 and 3). Values were normalized relative to EF, and calibrated to the levels in untransformed NIL-0f-1 plants. Error bars represent standard deviation of three biological replicates.

Additional file 3: RNA-seq data of ALS1, ALS2 and ALS3 genes. Expression level of ALS1, ALS2 and ALS3 genes from tomato, S. pimpinellifolium and potato in leaves and root (for tomato) or tuber (for potato) derived from RNA-seq data, and indicated as Fragments per Kilobase of exon per Million fragments mapped (FPKM) or Reads per Kilobase of transcripts per Million mapped (RPKM) values.

Additional file 4: Effect of exogenous application of amino acids on Oidium neolycopersici (On) fungal growth. Quantification of On fungal biomass, 8 days post inoculation of NIL-0f-1 plants sprayed with different amino acids solutions. Amino acids homoserine (HS), threonine (Thr), or branched-chain amino acids valine (Val), isoleucine (Ile) or leucine (Leu) were applied as described in Hublers et al. [10]. Data indicate the mean of three biological replicates with error bars representing the standard deviation. The asterisk indicates significant difference from the H2O control according to one way analysis of variance (P <0.05).

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

DG carried out the experiments. AEHML participated in the herbicide experiment. DG, RPH, and YB designed the experiments. YB drafted the final manuscript. DG, RPH, and YB designed the experiments. DG carried out the experiments. AEHML participated in the herbicide experiment. DG, RPH, and YB designed the experiments. YB drafted the final manuscript. RGFV commented on the manuscript. All authors read and approved the final manuscript.

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