A novel mutant allele of SSI2 confers a better balance between disease resistance and plant growth inhibition on Arabidopsis thaliana

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Abstract

Background: Resistance and growth are opposing characteristics in plants. SA INSENSITIVITY OF npr1-5 (SSI2) encodes a stearoyl-ACP desaturase (S-ACP DES) that has previously been reported to simultaneously enhance resistance and repress growth.

Results: Here, we characterize ssi2-2, a novel mutant allele of SSI2 that has two amino acid substitutions. Compared with wild-type and two other mutants of SSI2, ssi2-2 showed intermediate phenotypes in growth size, punctate necrosis, resistance to the bacterial pathogen Pst DC3000, salicylic acid (SA) content, pathogenesis-related (PR) gene levels and 18:1 content. These results indicate that ssi2-2 is a weak mutant of SSI2. Additionally, by using ssi2-2 as an intermediate control, a number of differentially expressed genes were identified in transcriptome profiling analysis. These results suggest that constitutive expression of defense-related genes and repression of IAA signaling-associated genes is present in all SSI2 mutants.

Conclusions: Taken together, our results suggest that the weak ssi2-2 mutant maintains a better balance between plant immunity and vegetative growth than other mutants, consequently providing a basis to genetically engineer disease resistance in crop plants.

Keywords: Auxin, Leaf shape, Plant immunity, Stearoyl-ACP desaturase, Salicylic acid

Background

Fatty acids (FAs) are crucial for all living organisms because they are not only a source of energy but are also major components of cellular membranes. Recently, an increasing number of studies has suggested that FAs and their derivatives have important roles as signaling molecules that modulate normal and disease-related processes [1]. In plants, FAs influence a variety of processes in response to both biotic and abiotic stresses [1, 2]. For example, the levels of polyunsaturated FAs in chloroplast membranes affect membrane lipid fluidity, which may affect plant tolerance to temperature stress [3, 4]. In addition, linolenic acid is involved in protein modifications in heat-stressed plants [5]. Azelaic acid, which is derived from C18 FA and contains a double bond at carbon 9, was shown to prime systemic acquired resistance (SAR) [6].

Stearoyl-ACP desaturase (SACPD) is a key enzyme that catalyzes the conversion of stearic acid (18:0) to oleic acid (18:1) during de novo FA biosynthesis and produces mono-unsaturated FAs in plant cells [7, 8]. The Arabidopsis thaliana genome has seven SACPD isoforms [8]. SSI2 is an SACPD isoform that can cause severe growth defects, including spontaneous necrosis and deformed leaves, when mutated. Analysis of plant pathogen resistance showed that the absence of SSI2 activates defense responses and leads to elevated salicylic acid (SA) levels and constitutive expression of pathogenesis-related (PR) genes, which results in enhanced resistance to several pathogens, such as Peronospora parasitica, Pseudomonas syringae pv. tomato (Pst) and...
Cucumber mosaic virus [9–17]. Others S-ACP-DES iso-
yzmes have greatly reduced specific activities compared to
SSI2, and knock-out mutations in S-ACP-DES 1 and 4 do
not alter defense phenotypes. The observations demon-
strate that SSI2 is the predominant SACPD isoform that
regulates defense signaling [9].

Using genetic approaches, an ssi2-1 suppressor was
isolated, namely act1, which encodes a glycerol-3-
phosphate (G3P) acyltransferase (ACT1). ACT1 is a key
enzyme that catalyzes the acylation of G3P with 18:1 to
form lysophosphatidic acid (lyso-PA). A mutation in act1
reduced the conversion of 18:1 to lyso-PA and recovered
the content of 18:1 in ssi2-1 mutants, which resulted in
growth restoration and reversion of the altered pathogen
response in ssi2-1 plants, suggesting that reduced 18:1
might be a direct cause of enhanced resistance and re-
tarded growth [13]. Additionally, restoration of the 18:1
levels can occur via second site mutations in G3P de-
hydrogenase (GLY1) [14] and acyl carrier protein 4 [15].

As chloroplastic 18:1 is distributed in the chloroplast,
the effect that 18:1 reduction has on nucleus-encoded
resistance genes remains elusive [9, 15, 16]. A possible
explanation is that the decreased 18:1 might lead to an
accumulation of NOA1 protein, which in turn acceler-
ates NO production and transcriptionally up-regulates
NO-responsive nuclear genes, thereby activating disease
resistance [18].

This 18:1-derived resistance appears to be conserved
among different plant species. Plants with a reduction of
SACPD isoforms showed increased resistance to patho-
genic bacteria and oomycetes in rice [19] and soybean
[20], respectively. Similarly, enhanced resistance to rice
blast was observed in rice OsSSI2 knockout mutants
[19]. This is not the case in Arabidopsis, as the ssi2-1
mutation enhanced resistance to powdery mildew [21]
but showed impaired resistance to B. cinerea [10]. In
addition to pathogen resistance, characterizing an
Arabidopsis fab2 mutant, which is a SSI2 null mutant
that differs from ssi2-1, revealed that SSI2 plays a role in
plant development and abiotic adaption, particularly to
high temperatures. Plants harboring fab2 mutations are
extremely small compared with wild-type plants [22, 23].
Microscopic analysis demonstrated that the fab2 muta-
tion lead to defective cell expansion in the mesophyll
and epidermal layers of leaves. Surprisingly, high tem-
peratures could ameliorate the severe growth defects of
fab2 mutants, which was not correlated with the fatty
acid composition. A possible explanation is that this re-
toration is due to increased membrane fluidity at higher
temperature [23].

Defense can be costly to the plant, and the expression of
defense genes can have negative effects on plant develop-
ment, which to some degree counterbalances their posi-
tive effects [24]. Lesion mimic mutants (LMM) always
have an altered plant form, such as snc1-1, lsd2, lsd4, dll1,
lrl1, which display reduced plant size. Further, PCD-
induced leaf necrosis may be correlated with the activation
of resistance responses [25]. Here, molecular analysis,
histological staining and transcriptional profiling reveal
that a weak mutation of SSI2 causes punctate necrotic
spots and enhanced resistance to the bacterial pathogen
Pst DC3000, with a degree of resistance less than that of
ssi2-1 and the T-DNA mutant, but the degree of growth
disruption was also reduced. This mutant thus provides
important information for potential genetic engineering to
improve disease resistance.

**Results**

ssi2-2 shows decreased growth and increased disease
resistance

To dissect the mechanism for plant immunity, we screened
for mutants that display significant LMM phenotype using
ethyl methanesulfonate (EMS)-mutagenized Arabidopsis
thaliana ecotype Columbia (Col-0). By close observation,
one mutant was found to have many small white spots on
its mature leaves and was isolated and designated ssi2-2
based on map-based cloning results (see below). Mature
ssi2-2 plants displayed growth defects, as shown by its small
and narrow leaves compared to those of wild-type plants
(Fig. 1a). To validate whether these white spots were due
to programmed cell death (PCD), we carried out trypan blue
staining, a widely used approach for selective detection of
dead tissues or cells. As expected, many blue spots were
found in mature leaves of ssi2-2, indicating that the white
spots result from PCD (Fig. 1b).

To test whether the ssi2-2 mutants have altered patho-
gen resistance, Pst DC3000 was inoculated into WT and
ssi2-2 mutant plants. The total number of bacteria in ssi2-
2 leaves was significantly lower than that in wild-type
leaves at 3 days post-inoculation (dpi), suggesting that
ssi2-2 exhibited activated resistance responses (Fig. 1c).
The transcriptional levels of several pathogenesis-related
genes, including PR1 (AT2G14610), PR2 (AT3G57260)
and PR5 (AT1G75040) were significantly up-regulated by
17.6-fold, 3.1-fold and 5.3-fold, respectively, in ssi2-2 com-
pared with wild-type plants (Fig. 1d). These results dem-
strate that ssi2-2 is involved in plant disease resistance.

**Cloning of ssi2-2 showed two nucleotide substitutions in
the SSI2 coding sequence**

To clone the gene, a segregating F2 population with ap-
proximately 6,000 individuals was generated. In this
process, we found that all F1 plants showed WT morpho-
logy, and all F2 seedlings exhibited a near 3:1 (134:43)
segregation of normal:narrow (WT:ssi2-2) leaf phenotypes,
indicating that ssi2-2 is caused by a single-gene recessive
mutation. Through rough mapping, the mutated gene was
located on chromosome II in a 3.4 Mb region between
NGA168 and CER461445. To facilitate fine mapping, new SSLPs and CAPS markers were developed in this region and 20 new polymorphic markers were generated. By using these newly developed markers, the mutated gene was finely mapped to an 83.8 kb region (Fig. 2a). We then designed 62 pairs of sequencing primers to re-sequence the entire candidate region. Compared to the reference sequence data of Col-0, two separate point substitutions were found in the coding region of SSI2 (AT2G43710), which led to amino acid alterations of A257T and R312H (Fig. 2b).

To establish a direct causal link with these mutations, an intact SSI2 genomic DNA fragment driven by the native SSI2 promoter was introduced into ssi2-2 plants. Normal growth size was observed in pSSI2::SSI2 transgenic offspring (Fig. 3a), confirming that growth defects of ssi2-2 are caused by SSI2 mutations. Because ssi2-2 has two mutations at the SSI2 locus, two different mutated SSI2 fragments, pSSI2::SSI2A257T and pSSI2::SSI2R312H (Additional file 1: Figure S1c), were also transformed into ssi2-2 mutants. Surprisingly, both constructs failed to complement the developmental and resistance phenotypes (Fig. 3a, b), demonstrating that both mutated amino acids in ssi2-2 are key for the functional SSI2 phenotype.

SSI2 encodes a stearoyl-ACP desaturase (S-ACP-DES) that catalyzes the production of oleoyl-ACP (18:1-ACP) from stearoyl-ACP (18:0-ACP) [10]. The previously characterized recessive mutant ssi2-1 lacks nearly 90% of this enzyme activity and exhibits pleiotropic phenotypes [10]. The ssi2-1 mutant was originally identified as a genetic suppressor of npr1-5 and exhibits constitutively activated plant defense responses without pathogen infection [26]. Based on these previous results and our current analyses, our newly isolated allele was renamed ssi2-2.
ssi2-2 shows intermediate phenotypes in development and disease resistance

To further study the function of SSI2, we analyzed ssi2-1 mutants [10] and a T-DNA insertion line (named ssi2-3) (Additional file 1: Figure S1a, b). Based on the sequence information in the TAIR database, the T-DNA insertion of ssi2-3 is located in the first intron of SSI2 (Fig. 4a). Obvious differences in phenotype were observed after cultivating these mutants in the growth chamber. The biomasses of ssi2-1 and ssi2-3 plants were 38.13 and 51.57 % compared to that of ssi2-2, respectively (Fig. 4b, c). Trypan blue staining showed that all three ssi2 mutants exhibited clear cell necrosis; differences in the necrotic areas were not noticeable among the three mutants (Additional file 1: Figure S2).

ROS burst is an important symbol of the plant disease resistance. Quantitation of ROS showed that the ssi2-2 mutant accumulated lower ROS level than the ssi2-1 and ssi2-3 mutants (Fig. 4d). Nitroblue tetrazolium (NBT) and 3, 3′-diaminobenzidine (DAB) staining detected accumulation of superoxide and peroxide, respectively. The staining patterns confirmed the model of ROS quantitation (Additional file 1: Figure S2). We used aniline blue staining to monitor callose deposition in leaf tissues. Bright blue staining in the mutants indicated that there was spontaneous callose deposition in the leaves of all three mutant lines (Additional file 1: Figure S2). These results are consistent with the known association between SSI2 and pathogen resistance.

In wild-type Arabidopsis plants, PR genes can be activated by various types of biotic stress. PRI is a marker gene for the SA signaling pathway in plants [27]. The ssi2-1, ssi2-2 and ssi2-3 mutants were capable of self-activating PRI expression, which showed increases of 245.5-, 29.5- and 88.9-fold, respectively, compared to the wild-type plants in the absence of exogenous SA, respectively, suggesting that SSI2 deficiency leads to constitutive activation of SA signaling. After treatment with exogenous SA, the expression of wild-type plants increased by 221.8-fold, while the ssi2-1, ssi2-2 and ssi2-3 plant expression levels increased by 3926.8-, 340.6- and 837.6-fold (Fig. 5a), respectively, indicating that PRI expression can be further up-regulated after SA treatment.

PDF1.2 and VSP2 can serve as marker genes for JA signaling [28]. The expression of PDF1.2 in ssi2-2 was similar to that of wild-type, but in ssi2-1 and ssi2-3 mutants, the expression of PDF1.2 was significantly reduced to 0.45- and 0.46-fold, respectively, of that of the wild-type. After treatment with exogenous JA, the expression of wild-type plants was up-regulated by approximately 4-fold, while that of the ssi2-1, ssi2-2 and ssi2-3 mutants increased by approximately 0.3-fold compared to the wild-type. A similar result was also observed in VSP2 gene expression analysis (Fig. 5b). These results are consistent with the northern blot results showing that several JA-inducible defense responses are impaired in ssi2-1 plants.

**Fig. 2** The ssi2-2 mutant harbors a new allele of SSI2. a Physical mapping of ssi2-2. Rough mapping showed that the ssi2-2 gene is located between markers NGA168 and CER461445 on chromosome 2 of Arabidopsis. Fine mapping revealed the gene between the primers of CAPS5 and CH2.1814, a range of 83.8 kb. The numbers below the molecular markers indicate the recombinant events detected between the ssi2-2 locus and the marker. b Structure of the ssi2-2 gene, AT2G43710. AT2G43710 encodes stearoyl desaturase and has three exons and two introns; sequence analysis revealed two point mutations in the third exon of the gene, namely G1952A and G2118A, resulting in changes in amino acids 257 and 312.
and demonstrating that the JA signaling pathway is also impaired in ssi2-2 and ssi2-3.

We also measured the SA content in mutant leaves. As shown in Fig. 5c, ssi2-1, ssi2-2 and ssi2-3 mutants had approximately 8-, 2- and 5-fold higher levels of SA than wild-type plants, respectively. Consistent with these results, all ssi2 mutants exhibited resistance phenotypes. The total amount of bacteria in wild-type leaves was 180.8-, 14.7- and 20.9-fold higher than that in ssi2-1, ssi2-2 and ssi2-3 leaves (Fig. 5d). In terms of resistance, the ssi2-1 mutant showed the strongest resistance, while the ssi2-2 mutant had the weakest resistance.

SSI2 is located in the chloroplast. We also determined the subcellular location of SSI2 in the mutant ssi2-2 and SSI2A257T SSI2R312H mutants. Similar to the WT, a protein with two mutated amino acids (ssi2-2) or single amino acid mutations (SSI2A257T, SSI2R312H) was located in the chloroplast (Additional file 1: Figure S3), indicating that point mutations in ssi2-2 did not affect the subcellular localization of the protein.

SSI2 regulated the expression of genes involved in SA and IAA pathways

To elucidate the mechanism underlying the observed phenotype of ssi2 mutants, an RNA sequencing experiment was conducted (Additional file 2: Table S1). Compared to wild-type, thousands of genes displayed significant changes
in transcript levels in all mutants. Specifically, the ssi2-2 mutant had 1,527 up-regulated genes and 6,422 down-regulated genes. The total number of up-regulated genes (5,896 and 3,275) and down-regulated genes (3,067 and 4,105) was identified in ssi2-1 and ssi2-3 mutants, respectively (Fig. 7a).

Because all three mutants showed increased resistance to Pst DC3000 and reduced growth size, after integrated analysis of the RNA-seq data, the common 747 up-regulated genes and 892 down-regulated genes were identified in all three mutants. Using the ssi2-2 mutant as an intermediate control, 484 of the 747 up-regulated genes showed reduced expression in the ssi2-2 mutant compared to the ssi2-1 and ssi2-3 mutants, in accordance with the “weak mutant” phenotype. GO annotations were assigned to the differentially expressed genes in the mutant lines, and PANTHER [29] analysis revealed a significant enrichment in immune system processes and SA signaling pathways among these
genes (Additional file 2: Table S2), which is consistent with the previous conclusion that the SA pathway is associated with low levels of oleic acid. To validate the data of the RNA-seq experiments, the expression levels of *PAD4* (AT3G52430), *EDS5* (AT4G39030), and *ICS1* (AT1G74710) were tested by quantitative RT-PCR and exhibited the same expression pattern (Fig. 7b). In addition, genes categorized as response to chitin (GO:0010200) and response to nitrogen compounds (GO:1901698) were significantly enriched in the analysis, implying that *SSI2* also mediated additional resistance responses.

We tried to analyze the molecular mechanism controlling the developmental phenotype of the *ssi2* mutants. Abnormal growth phenotypes are often closely related to plant hormones. By agriGO [30] analysis, for the hormone-related GO annotation, the highest degree of enrichment of the 892 down-regulated genes was response to auxin stimulus (GO:0009733) (Fig. 8a), suggesting that reduced oleic acid may inhibit auxin-mediated pathways, affecting the developmental process. Further analysis identified that
24 genes out of 892 genes were auxin-related (Additional file 2: Table S3). By using quantitative RT-PCR, 10 out of 24 auxin-related genes showed less repression in the ssi2-2 mutant than in the ssi2-1 and the ssi2-3 mutants. Notably, SAUR20 (AT5G18020), SAUR21 (AT5G18030), SAUR22 (AT5G18050), SAUR23 (AT5G18060), and SAUR24 (AT5G18080) belong to a subgroup of SMALL AUXIN UP RNA (SAUR) genes which have been reported to be connected with plant leaf size [31]. Another two SAUR proteins, SAUR61 (AT1G29420) and SAUR62 (AT1G29430), play a role in organ development [32]. IAA5 (AT1G15580) and IAA11 (AT4G28640) as two early auxin-induced transcription factors, were also significantly repressed in SSI2 mutants. These results imply that the auxin mediated pathway combines with the SSI2-mediated pathway to produce the narrow-leaf dwarf phenotype.

**Discussion**

The ssi2-1 mutant was previously identified as being resistant to pathogens, and the ssi2-1 npr1 double mutant was susceptible to *Pst* DC3000. It was suggested that resistance to *Pst* DC3000 which was mediated by ssi2-1, depended on NPR1. However, depletion of NPR1 could not suppress the increased SA content, development of spontaneous necrotic spots and constitutively high expression of *PR* genes in ssi2-1, demonstrating that SSI2 also mediates a NPR1-independent pathway [26]. The ssi2-2 mutant also displayed constitutive expression of *PR* genes (Fig. 1d, Additional file 2: Table S2), spontaneous necrosis and pathogen resistance, demonstrating that ssi2-2 is a novel allele of SSI2. Applying exogenous SA to ssi2 mutants increased the expression of *PR* genes. These results, combined with the fact that SSI2 mutants are only resistant to biotrophic pathogens and are highly susceptible to necrotrophic pathogens, suggest that SSI2-mediated resistance is principally dependent on the SA pathway. Mutations in SSI2 may regulate the SA signaling pathway, but are not necessarily involved in this pathway, and SSI2 functions upstream of the *EDS1* gene [12].

Compared to the ssi2-1 and ssi2-3 mutants, the overall growth of ssi2-2 plants was significantly higher (Fig. 4b, c). ssi2-2 carries two amino acid substitutions that are not located in the central activated region of the enzyme. The improved growth might be explained by higher enzymatic activity in ssi2-2 plants compared to ssi2-1 and ssi2-3 plants which is supported by a smaller decrease in oleic acid in ssi2-2 plants (Fig. 6). Several indicators of disease resistance, such as the leaf ROS levels, *PR* gene expression levels and number of bacterial colonies after infection, were also intermediate in the ssi2-2 mutant, between those of wild-type plants and ssi2-1 and ssi2-3 mutants (Figs. 4 and 5). These characteristics support the conclusion that ssi2-2 is a new variant.
weak mutant allele of SSI2. By sequence alignment, the corresponding site of Arabidopsis Ala^{257} is Ala^{220} in castor, which is close to the pairs of iron binding helices [33]. Iron binding plays a key role in interrupting the C-H bond of the fatty acid chain [34]. Does the A257T mutation in Arabidopsis interfere with iron binding, thereby reducing enzyme activity? It remains unclear. Via subunit structure analysis in castor, Arg^{274}, which corresponds with Arg^{312} in Arabidopsis, is predicted to interact with Asp^{358} after the eighth α-helix and before the second β-hairpin [33]. Therefore, the R312H mutation in ssi2-2 was hypothesized to interfere with the structure of SSI2, resulting in decreased enzyme activity.

Decreased enzymatic activity alone seems insufficient to explain SSI2-mediated resistance. The 18:1 content was similar between ssi2-1 and ssi2-3 plants, but the SA content in ssi2-1 leaves was much higher than in ssi2-3 leaves, and the resistance of the ssi2-1 mutant was also obviously stronger than that of the ssi2-3 mutant. One explanation is that SSI2 interacts with other proteins or macromolecules, that are necessary for SSI2-mediated signaling.

What is the relationship between reduced oleic acid levels and disease resistance? In expression profiling analysis, we focused on two specific enriched GO items (response to chitin GO:0010200 and response to nitrogen compound GO:1901698), considering the facts that 18:1 regulates NO production in the chloroplast [18] and chitin is regarded to be a typical pathogen-associated molecular patterns (PAMP) [35]. Lower 18:1 levels perhaps not only regulated the downstream SA signaling pathway but also acted as an earlier signal in the plant resistance response.

We also observed that many auxin-related genes showed altered transcriptional levels in the mutants (Additional file 2: Table S3). Specifically, some SAUR and IAA genes were down-regulated and confirmed by qPCR (Fig. 8b), suggesting that these IAA response genes are probably involved in the regulation of leaf development in SSI2 mutants. Generally, elevated salicylic acid inhibits pathogen growth by through repression of the auxin signaling pathway [36]. Because SSI2 mutants activated SA and other disease resistance signaling pathways, these pathways may have antagonized the IAA signaling pathways and regulated SAUR-mediated developmental signaling by an unknown mechanism.

**Conclusions**

Previous studies have demonstrated the genes involved in photosynthesis and growth were down-regulated during induced resistance [37]. However, few studies have focused on the costs and trade-offs associated with induced resistance to pathogens. The limiting effect of
disease resistance on yield should continue to be studied. The ssi2-1 and ssi2-3 mutants show dwarf phenotypes, and SACPD gene silencing significantly reduced soybean plant height and seed yield [20]. These results demonstrate that high disease resistance often comes at a great cost to plants. Accordingly, the weak ssi2-2 mutant presented a better balance between resistance and growth which could be a great advantage in crop breeding, especially in plants with lower fatty acid requirements, such as vegetables and trees, in which pathogen resistance could be gained with little effect on growth. For crops with stricter requirements in terms of fatty acid composition, such as canola and corn, we could also develop a strategy to breed lines that have leaves that are low in oleic acid, but that have seeds that contain an unchanged fatty acid composition to achieve a balance between plant growth and pathogen resistance. Our research therefore provides a theoretical basis for choosing effective resistance breeding strategies.

Methods
Plant cultivation
ssi2-1 mutant seeds were kindly provided by Prof. Kachroo, and SALK_039852 (ssi2-3) seeds were obtained from The Arabidopsis Information Resource (http://www.arabidopsis.org). Seeds of the Arabidopsis Col-0 and Ler-0 ecotypes and other mutant lines were first surface-sterilized with 5 % (v/v) sodium hypochlorite and then thoroughly washed six times with sterile water. After vernalization at 4 °C for 2 d in darkness, Arabidopsis seeds were grown in soil or on 1/2 Murashige and Skoog (MS) medium containing 1 % (w/v) sucrose and cultured in a growth chamber. The growth chamber was controlled at an irradiance of 120 μmol quanta m$^{-2}$ sec$^{-1}$ at 22 °C with 85 % relative humidity under 12 h light and 12 h dark cycles. A nutrient solution was supplied with water every 3 days to sustain plant growth.

Mutant screen and map-based cloning
Approximately 30,000 M$_2$ plants were screened at 22 °C for lesion mimic phenotypes. To isolate the ssi2-2, a homozygous mutant plant was first crossed with Ler-0 to generate F$_1$ progeny, which in turn were self-pollinated to produce F$_2$ progeny. Bulked segregation analysis was performed on pools of 20 plants with simple sequence length polymorphisms (SSLPs) by PCR amplification, and the 1,200 narrow-leaf plants were used for genetic mapping by PCR amplification of SSLPs. SSLPs and derived cleaved amplified polymorphic sequence (CAPS) markers between the Col-0 and Ler-0 ecotypes were used for fine mapping. Primers were designed with (http://helix.wustl.edu/dcaps/dcaps.html). The primers used in map-based cloning are listed in Additional file 2: Table S4.

Generation of transgenic plants
For the pSSI2::SSI2 transgenic line, a 4.2-kb genomic fragment containing the SSI2 promoter region and coding sequence was amplified by PCR from the wild-type (Col-0) and inserted into the pCXGFP-P [38] vector by TA cloning. We generated the single mutation AtSSI2 transgenic construct with PCR-based mutation using the Fast Mutagenesis System (FM111-01, Transgen Biotech, Beijing, CN). The primers for vector construction are listed in Additional file 2: Table S5. The binary vector was transformed into ssi2-2 plants using the floral dip method [39]. Transgenic plants were selected on plates containing hygromycin. The complementation test was confirmed by genotypic analysis of the T$_2$ plants.

Quantitative RT-PCR analysis
Total RNA was isolated from 100 mg plant tissue with TRI reagent according to the manufacturer’s instructions (T9424, Sigma-Aldrich, USA). RNA (0.5 μg) was used for first-strand cDNA synthesis with a PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa, Dalian, CN). Quantitative PCR was performed with SYBR® Premix Ex Taq™ (Tli RNaseH Plus) on an IQ5 Real-Time PCR System (Bio-Rad, USA). The PCR was performed as previously described [40]. AtACTIN2 of Arabidopsis was used as an internal control to standardize the results. For each gene, qRT-PCR assays were repeated at least twice with triplicate runs. The relative expression levels were determined using the 2$^{-ΔΔCt}$ analysis method. The sequences of the primers for all of the detected genes are listed in Additional file 2: Table S5.

Plant disease resistance assay
For disease resistance assays, 28-d-old plant leaves were sprayed with virulent Pseudomonas syringae pv. tomato DC3000 at optical densities at 600 nm of 0.2. Bacterial cultures were grown overnight in King’s B medium containing rifampicin and/or kanamycin. Inoculation with 10 mM MgCl$_2$ was used as a mock treatment. Inoculated plants were covered with a clear plastic dome to maintain humidity throughout the course of the experiment. At 0 and 3 dpi, the treated leaves were harvested. The leaves were homogenized in 10 mM MgCl$_2$, diluted 10$^3$- or 10$^4$-fold, and plated on King’s B medium. P. syringae-related experiments were repeated three times for every genotype analyzed.

DAB, NBT, trypan blue and aniline blue staining
DAB staining and NBT staining were performed as previously described [41]. Briefly, the seedlings were immersed in DAB solution (1 mg mL$^{-1}$) and NBT solution (1 mg mL$^{-1}$) at room temperature for 8 h. The stained seedlings were then transferred to 70 % (v/v) ethanol to remove chlorophyll and visualize brown
and blue spots, which represented $H_2O_2$ and $O_2$, respectively. Leaves of 28-d-old plants were stained in a lactophenol trypan blue solution as described previously [42]. Ethanol (70 % v/v) was used to remove the chlorophyll, and leaves were then photographed. Callose depositions were visualized using aniline blue (0.01 % in 150 mM $K_2HPO_4$, pH 9.5) as described previously [26]. Stained leaves were stored in a 50 % glycerol solution in the dark and subsequently examined for fluorescence using a Nikon 90I microscope with a standard filter block for ultraviolet fluorescence UV-2A (excitation 330–380 nm).

**ROS quantitation**

The ROS level was determined using 2, 7-dichlorodihydro-fluorescein diacetate (H$_2$DCF-DA) (Beyotime Institute of Biotechnology, Haimen, China). The leaves were directly treated with 10 mM H$_2$DCF-DA dissolved in PBS at 37 °C for 30 min. The epidermis was isolated and the fluorescence intensity was monitored with an excitation wavelength at 488 nm and emission wavelength at 525 nm. Quantification of ROS was performed using ImagePro Plus software as previous study [43].

**Leaf fatty acid (FA) analyses and SA quantification**

FA analysis was performed as previously described [16]. Briefly, FA extraction was carried out by placing leaf tissue in 2 mL of 3 % sulfuric acid in methanol. After a 30 min incubation at 80 °C, 1 mL of hexane with 0.001 % butylated hydroxytoluene was added. The hexane phase was then transferred to vials for gas chromatography (GC). The SA concentration was quantified by GC-MS analysis according to Brader et al. [44].

**Fluorescence microscopy analysis**

GFP was used as a reporter to investigate the subcellular localization of different gene allele fragments in planta. The full-length SSI2 gene and three mutation fragments were cloned into vector pEarley103 (Invitrogen, CA, USA). The constructs were introduced into the A. tumefaciens strain GV3101 and transiently expressed in the tobacco epidermis. Live plant imaging was performed on a Zeiss LSM510 META confocal microscope (Carl Zeiss) using a 40× C-Apochromat water immersion objective lens.

**Transcriptional profiling**

Total RNA was isolated from 4-week-old plants using TRI reagent. RNA was used to create a cDNA library for sequencing on an Illumina HiSeq™ 2000. RNA sequencing was performed with the commercial service from Chinese National Human Genome Center at Shanghai (CHGC, www.hanyubio.com). The DEGseq package with MARS (MA plot-based method with random sampling model) [45] was used to analyze the data. A sequence data set comprising Arabidopsis Col-0 unigenes from the TAIR database (http://www.arabidopsis.org) was used as the reference gene set for read mapping. GO term annotations and WEGO were used to classify their putative functions. Differences in gene expression were calculated for each contig in every sample-pair. FDR values of less than 0.001 and a signal strength log$_2$ ratio ≥ 1 or ≤ −1, i.e., 2-fold increase or decrease, respectively, were used as the cut-offs for significant differences.

**Data treatment**

The biomass data were analyzed by one-way ANOVA followed by the Tukey test. Quantitative data were analyzed by Student’s t test (two-tail t test with equal variances; Microsoft Excel) to determine the significant differences between wild-type and mutant plants. $P < 0.05$ is indicated by an asterisk, and $P < 0.01$ is indicated by two asterisks.

**Additional files**

- **Additional file 1: Figure S1.** Mutant test and transgenic plant confirmation. Figure S2. Cytological staining of Col-0 and different ssi2 mutant lines. Figure S3. Quantitative RT-PCR analysis of 14 auxin related genes. Figure S4. Subcellular localization of SSI2 and mutant fragments. (PPTX 1923 kb)
- **Additional file 2: Table S1.** EXCEL file with RNA-seq data. Table S2. GO analysis of the 484 up-regulated genes. Table S3. Transcriptional profiling data for several SA and IAA pathway genes. Table S4. Primers used in map-based cloning. Table S5. Primers used in the vector construction and mutant analysis. (XLSX 7942 kb)

**Abbreviations**

CAPS: Cleaved amplified polymorphic sequence; FA: Fatty acid; FAB2: FATTY ACID BIOSYNTHESIS 2; NPR1: NON-EXPRESSION OF PATHOGENESIS-RELATED GENES 1; PAMP: Pathogen-associated molecular patterns; PDF1.2: PLANT DEFENSIN 1.2; PR1: PATHOGENESIS-RELATED 1; qRT-PCR: Quantitative reverse transcription-PCR; SA: Salicylic acid; SAR: Systemic acquired resistance; SS2: SUPPRESSOR OF SA INSENSITIVITY 2; SSLP: Simple sequence length polymorphism

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**Availability of supporting data**

The data sets supporting the results of this article are included within the article and its additional files. The nucleic acid sequence of ssi2-2 is available in NCBI’s database with accession number KU950361 (http://www.ncbi.nlm.nih.gov/ nucleotide). The RNA-seq data supporting the results of this article are available in the NCBI’s SRA with the accession number SRP072265 (http://www.ncbi.nlm.nih.gov/sra).
Authors’ contributions
ZC and XD designed the experiments. WY, RD, LL, ZH and JL performed the experiments. WY, RD, YW and XD analyzed data. WY, LL, ZH and ZC co-wrote the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Not applicable.

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