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## *Arabidopsis thaliana* SKP1 interacting protein 11 (At2g02870) negatively regulates the release of green leaf volatiles†

Muhammad Naeem-ul-Hassan,<sup>ID</sup>\*<sup>ac</sup> Zamri Zainal,<sup>ab</sup> Chew Jin Kiat,<sup>ab</sup> Hossein Hosseini Monfared<sup>a</sup> and Ismanizan Ismail<sup>\*ab</sup>

Green leaf volatiles (GLV) are important factors in plant defense, and plant–plant and plant–insect interactions. Kelch-repeat containing F-box proteins (KFB) are plant-specific proteins, which recruit substrates for protein degradation. The present study was conducted to evaluate the role of AtSKIP11, an *Arabidopsis* KFB, in the expression of hydroperoxide lyase (HPL) pathway genes and the formation of products by creating transgenic *Arabidopsis thaliana* plants expressing the AtSKIP11 open reading frame and its antisense sequence. We found that production of C5 volatiles was greatly reduced in these transgenic plants. The production of C6 GLV was reduced by 49% in AtSKIP11 overexpressing plants, whereas the production of these compounds was increased by 12% in the AtSKIP11-antisense suppression plants. Real time quantitative polymerase chain reaction (RT-qPCR) results showed that expression of *LOX2*, *HPL* and *ADH1* genes was reduced by 1.8, 1.3 and 2.5 fold, respectively, in the AtSKIP11 overexpressing plants, compared with wild-type plants. In the AtSKIP11-antisense plants, expression of *LOX2* was reduced 1.2 fold, but *HPL* and *ADH1* genes were up-regulated by 1.2 fold and 1.7 fold, respectively, over the wild-type samples. Our results suggest the involvement of AtSKIP11 in negatively regulating the HPL pathway, especially the expression of *ADH1*, possibly through degradation of the transcription factors.

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## Introduction

The sessile nature of the plant kingdom does not allow these creatures to move towards a safe or a favourable place. Moreover, these organisms lack the specialized immune system animals use to combat diseases. Alternatively, plants have developed various other mechanisms to combat a variety of threats. One way of doing this is through inducing the production of defense-related proteins and the release of toxic chemicals. These chemical defenses are the products of the secondary metabolism of plants and they obstruct the growth of and potential damage by different pathogens, including viruses, fungi, bacteria and herbivores. Thousands of different kinds of secondary metabolites are synthesized in plants by a large number of inducible pathways. The oxylipin pathway is among the major inducible pathways for the production of defense

responses through activation of its genes in response to different biotic and abiotic stresses.<sup>1,2</sup> These stresses can activate the oxylipin pathway for the release of higher amounts of jasmonates and green leaf volatiles (GLV), which are otherwise released in very low concentrations under normal conditions.<sup>3</sup>

Plant oxylipins constitute a diverse class of bioactive lipids that are produced by the oxidation of  $\alpha$ -linolenic acid (ALA, 18 : 3 $\Delta$  (ref. 9, 12 and 15)) and linoleic acid (LA, 18 : 2 $\Delta$  (ref. 9 and 12)). Products of the oxylipin pathway include 12-oxo-phytodienoic acid, jasmonic acid (JA), methyl jasmonate (MeJA), and GLV.<sup>4–6</sup> Although jasmonates are well known for their role in the regulation of many physiological processes in plant development, they are particularly regarded as specialists for host immunity and plant defense.<sup>7–9</sup> The production of GLV is induced under conditions of stress in order to protect the plants against herbivores and microbial pathogens.<sup>10</sup> Under normal physiological conditions, only traces of GLV are released. However, the production of these compounds abruptly increases in response to tissue disruption resulting from biotic or abiotic stresses.<sup>3</sup>

The hydroperoxide lyase (HPL) branch of the oxylipin pathway takes its share from the polyunsaturated fatty acids (PUFA) hydroperoxide substrates, generated by lipoxygenase (LOX) enzymes due to activation of the pathway genes under the stressed conditions.<sup>11</sup> The products of LOX are divided between the two competing branches of the oxylipin pathway in

<sup>a</sup>School of Bioscience and Biotechnology, Faculty of Science and Technology, University Kebangsaan Malaysia, Malaysia

<sup>b</sup>Institute of Systems Biology (INBIOSIS), University Kebangsaan Malaysia, Bangi, 43600, Selangor, Malaysia. E-mail: maniz@ukm.edu.my; ismanizan@gmail.com; Tel: +60 389214546

<sup>c</sup>Department of Chemistry, University of Sargodha, Sargodha, Pakistan. E-mail: sobheel@yahoo.com; Tel: +92 3338967518

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a coordinated manner.<sup>12,13</sup> Allene oxide synthase converts these intermediates to jasmonates and the HPL enzyme releases volatile C9 and C6 aldehydes. These aldehydes are further converted to other aldehydes, alcohols and their acetylated derivatives.<sup>14</sup> Among the HPL downstream enzymes are included (3Z):(2E)-hexenal isomerase, responsible for the conversion of Z-3-hexenal to its E-2 isomer, and NAD-dependent alcohol dehydrogenase (ADH), that catalyzes the reduction of aldehydes to their respective alcohols in order to confer higher stability.<sup>15–17</sup> All these saturated and unsaturated aldehydes and their downstream products synthesized in the plant's vegetative tissue are volatile in nature and are collectively named as GLV. The role of GLV in the plant's response to various stresses, like microbial pathogens and herbivory attack, has been studied since the early 1990s. But they have been getting the special attention of researchers around the world for the last decade and a half because of their emerging role as aerial messengers for plant–plant communication.<sup>18,19</sup>

The regulation of cellular processes through the degradation of regulatory proteins by the ubiquitin 26S proteasome system (UPS) followed by recognition *via* specific F-box proteins is a common signaling mechanism. F-box is an emerging family of signaling proteins in eukaryotes and is thought to be one of the largest superfamilies of plant proteins.<sup>20</sup> The majority of F-box proteins serve as a substrate-recruiting component of SCF (SKP1, Cullin 1, RING-BOX 1 and an F-box protein) complexes. Nearly two decades since the recognition of and giving the name 'F-box' to a domain in cyclin-F protein, our understanding of the role of F-box proteins, SCF biology and the molecular mechanisms employed by UPS is steadily developing.<sup>21</sup> F-box proteins are comprised of a conserved 40–50 amino acids F-box domain on the N-terminal, while the C-terminals contain one or more secondary motifs. These motifs, which include WD40-repeats, leucine rich-repeats (LRR), kelch-repeats and some other motifs, may be the same or variable in a particular protein and are involved in protein–protein interactions.<sup>22,23</sup> Researchers around the world have identified many F-box proteins since the discovery of cyclin-F, especially in plants. Since hundreds of SCF complexes have been characterized, the number of F-box proteins serving these SCF complexes is also quite large. However, most of these F-box proteins either remain 'orphans' or, until now, could not be completely characterized for their function.<sup>24</sup>

F-box proteins containing the kelch-repeat motifs (KFB) are among the major plant F-box proteins and are considered to be plant specific. The fact that only a couple of KFB have been characterized in non-plant organisms suggests that the KFB subfamily of F-box proteins is exclusively a plant-specific family of genes.<sup>25,26</sup> This indicates that some very crucial roles have been assigned to these proteins in the plant cell. In recent years, the pace of research involving plant KFB in molecular signaling has advanced at a reasonable rate, but there is still a lot more to be explored. Several KFB have been reported for their signaling roles in plant defense, plant physiology, circadian mechanisms, secondary metabolism, growth and development, but still the majority of these proteins remain 'orphans' or under investigation.<sup>24</sup>

Although several recent studies have highlighted the role of different KFB in plant physiology, not even a single KFB has been reported for any role in the HPL branch of the oxylipin pathway.<sup>27–29</sup> A couple of recent studies identified and characterized a novel KFB gene in *Persicaria minor*, named *PmF-box1*. This gene was found to respond to jasmonic acid elicitation and co-up-regulated with *PmADH1* and *PmLOX1* upon exogenous treatment with jasmonic acid.<sup>30,31</sup> BLAST searches were run to find a *PmF-box1* homolog in *Arabidopsis thaliana*. The results returned by the BLAST search show that SKP1 interacting protein 11 (*SKIP11*) possesses the highest (69%) sequence similarity with *PmF-box1*. AtSKIP11 (At2g02870) is a KFB consisting of 467 amino acid residues with five kelch repeats. Although no specific function has been identified for AtSKIP11 until now, the protein has been found to interact with the *Arabidopsis thaliana* ASK1 and ASK2 proteins.<sup>29,32</sup> The present study highlights the role of the *AtSKIP11* gene in the HPL branch of the oxylipin pathway for the production of GLV. A full length *AtSKIP11* gene sequence was cloned to the pB2GW7 gateway destination vector to form overexpression and antisense suppression constructs. Both the constructs were transferred to *Arabidopsis thaliana* wild-type (At-WT) plants by *Agrobacterium tumefaciens* mediated transformation using the floral dip method. Functional analysis was performed on the T2 generation of the transgenic plants, along with the At-WT plants (control plants). Our results have shown that the *AtSKIP11* overexpressing plants produced a lower amount of GLV as compared to the wild-type *Arabidopsis* plants. Whereas, the production of GLV was found to be enhanced in the transgenic *Arabidopsis* plants in which the *AtSKIP11* gene transcript was suppressed by the antisense strategy. These results suggest that *AtSKIP11* is involved in the biosynthesis of GLV, possibly affecting the transcription factors, and negatively regulates the HPL pathway.

## Experimental

### Plant material and bacterial cultures

*A. thaliana* ecotype Columbia-0 seeds were obtained from Dr Noor Azmi Shaharudin (Universiti Putra Malaysia). Chemically competent *E. coli* strain TOP10 cells and the GV3101 strain of *A. tumefaciens* were obtained from Plant Biotechnology Lab, Universiti Kebangsaan Malaysia.

### Chemicals and reagents

All the chemicals and reagents used in this study were of analytical grade or higher. The chemicals used for the molecular work and as the reference standards were obtained from Sigma-Aldrich (USA). For the tissue culture work, chemicals were obtained from Duchefa Biochemie B.V. (Netherlands). The QIAquick Gel Extraction Kit and the DNeasy Plant Mini Kit were from QIAGEN (UK). The Gateway Cloning kit, PureLink Quick Plasmid Miniprep Kit, Plant RNA Reagent, Platinum *Taq* DNA Polymerase, Ambion TURBO DNase and DNA Ladders were obtained from Life Technologies (Carlsbad, CA, USA). KAPA SYBR FAST qPCR Kit, KAPA *Taq* and KAPA *Taq* HotStart DNA



Polymerase were from Kapa Biosystems (Wilmington, Massachusetts). Restriction enzymes and the ProtoScript First Strand cDNA Synthesis Kit were from New England Biolabs (MA, USA).

### Construction of *AtSKIP11* overexpression and antisense recombinant plasmid

#### a. Isolation of *AtSKIP11* gene and its antisense sequence.

The nucleotide sequence of *A. thaliana* F-box protein, *AtSKIP11* was retrieved from the NCBI database with the GenBank ID At2g02870. About 4–5 healthy leaves were cut from the *A. thaliana* plants and the total RNA was extracted by PureLink Plant RNA Reagent and treated with a turbo DNA-free kit from Life Technologies according to the manufacturer's protocol. The cDNA was synthesized by a ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs), according to the kit manufacturer's protocol.

For the cloning of the *AtSKIP11* gene and its antisense sequence, the full length open reading frame was amplified by a standard PCR reaction, using *A. thaliana* cDNA as the template. The primers for the isolation of the *AtSKIP11* gene with flanking attB sites were designed as guided by the Gateway Cloning system and verified by the OligoAnalyzer 3.1 tool from Integrated DNA Technologies (IDT). Using these primer pairs, fragments of 1555 bps were amplified by PCR for each *AtSKIP11* open reading frame and its antisense sequence plus Gateway attB sites (Table 1).

The PCR reaction was run with Platinum Taq DNA Polymerase from Life Technologies using *Arabidopsis* cDNA as the template. Two separate 50  $\mu$ L PCR reactions were performed on an Eppendorf Mastercycler machine with the following program: initial denaturation of 1 cycle at 94 °C for 2 min; and 30 cycles of: denaturation at 94 °C for 15 s, primers annealing at 55 °C for 30 s, extension at 68 °C for 1 : 30 min and the final hold at 10 °C. The PCR products were analyzed by 1% (w/v) agarose-EtBr gel electrophoresis and purified by a Purelink PCR purification kit (Life Technologies) according to the manufacturer's protocol. The quality of the purified PCR product was verified by NanoDrop and then stored at –20 °C until the next step.

**b. Cloning of *AtSKIP11* gene and *AtSKIP11*-antisense in the gateway destination vector.** The attB sites containing PCR products were cloned into pENTR221, using 'BP Clonase II' enzyme mix (Life Technologies) to yield the entry clones pENTR::*AtSKIP11* and pENTR::*AtSKIP11*-antisense. LR clonase reactions were then carried out to transfer the *AtSKIP11* and the *AtSKIP11*-antisense cDNA fragments from the respective entry clones to the gateway destination vector, pB2GW7. Expression

clones, pB2GW7::*AtSKIP11* and pB2GW7::*AtSKIP11*-antisense, were constructed as a result of these reactions. Successful transformants were screened by LB agar-spectinomycin selection and verified by colony PCR using the gene-specific primers given in Table 1. Further confirmation of the integration of the *AtSKIP11* gene and the *AtSKIP11*-antisense sequence in the recombinant plasmids was accomplished by RE digestion analysis and sequencing after extracting the plasmids from the positive clones.

### Generation of transgenic *Arabidopsis thaliana* plants

*Agrobacterium tumefaciens* strain GV3101 harbouring pB2GW7::*AtSKIP11* and pB2GW7::*AtSKIP11*-antisense recombinant plasmids was used to transform the *A. thaliana* (ecotype Columbia) plants by the floral dip method.<sup>33</sup> The plants were grown under standard conditions and selected by the herbicide 'glufosinate' (basta) due to the presence of the *bar* gene in the recombinant plasmids. The putative transgenic plants, which survived the herbicide treatment, were confirmed for the integration of the transgenes by PCR, using the genomic DNA as the templates. The PCR was run with Kapa Polymerase Master Mix and the primers listed in Table 2.

The PCR Master Mix was prepared as given in the manufacturer's manual. The following PCR program was employed: 1 cycle of initial denaturation at 95 °C for 3 min; and 30 cycles of: denaturation at 98 °C for 30 s, primers annealing at 62 °C for 30 s, primers extension at 72 °C for 1 : 30 min and 1 cycle of final extension at 72 °C for 3 min. The products of PCR were analyzed by running 1% agarose-EtBr gel electrophoresis and comparing the bands with a 1 kb DNA ladder to confirm the integration of the DNA insert.

### Molecular analysis of transgenic *A. thaliana*

Molecular analysis of transgenic *A. thaliana* plants was initiated by growing the third generation. Three lines were selected from each type of transgenic plant for the analysis of metabolic products and the gene expression of the HPL pathway. To ensure the growth of only the desired transgenic plants and to avoid the growth of any non-transgenic contaminants, the young seedlings were again subjected to the Basta selection. Confirmed transgenic plants were grown in the growth chamber until six weeks of age under standard conditions before sampling. Two hours prior to sampling, the plants were treated with 250  $\mu$ M methyl jasmonate aqueous solution to activate the plant defence related gene expression and the production of plant defence compounds. After two hours the samples were

**Table 1** Primer sequence used to prepare the cDNA insert in the preparation of construct pB2GW7::*AtSKIP11* and pB2GW7::*AtSKIP11*-antisense

Primer	Sequence (5'–3')	Amplicon size
At-SKIP11-attB1 (F)	GGGGACAAGTTTGTACAAAAAGCAGGCTGGGTTTGGGATTGGGATTGATCTGA	1555 bps
At-SKIP11-attB2 (R)	GGGGACCACTTTGTACAAGAAAGCTGGGTGGAGGGTCTTCAATGGATAGTCGT	
At-SKIP11-attB1 antisense (F)	GGGGACAAGTTTGTACAAAAAGCAGGCTGGAGGGTCTTCAATGGATAGTCGT	
At-SKIP11-attB2 antisense (R)	GGGGACCACTTTGTACAAGAAAGCTGGGTGGGTTTGGGATTGGGATTGATCTGA	



**Table 2** Primers for confirmation of transgenes in putative transgenic *Arabidopsis thaliana*

Primer	Sequence (5'–3')	Amplicon size
b2sk-F1 (pB2GW7 specific)	CCGTGAAGACTGGCGAACA	
b2skox-R1 ( <i>AtSKIP11</i> specific)	GCCAGCTCTTTCAGGCAATCTT	≈ 1830 bps
b2skanti-R1 ( <i>AtSKIP11</i> -antisense specific)	CCGATATGGAAGTGAGGAAGTACGA	≈ 960 bps

collected for real-time quantitative PCR (RT-qPCR) and solid phase micro-extraction GC-MS (SPME-GC-MS) analysis.

### RT-qPCR analysis

Traces of any methyl jasmonate, sprayed two hours before, were adsorbed from the leaf surface using a clean tissue paper. The upper parts of 4–5 healthy leaves (≈ 300 µg) were cut with a clean pair of scissors and the plant material was quickly transferred to labelled 1.5 mL RNase free microfuge tubes and immediately frozen in liquid nitrogen. Total RNA was then extracted by using the Purelink Plant RNA Reagent (Life Technologies, USA) according to the manufacturer's instructions. After removing any contaminating DNA, first strand cDNA synthesis was accomplished by a ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs) using 500 ng of purified total RNA as the template.

Analysis for the expression of *Arabidopsis Actin2* (*Act2*), *LOX2*, *HPL* and *ADH1* genes was performed by RT-qPCR, using a KAPA SYBR FAST qPCR Kit (Kapa Biosystems, USA). For each gene, a pair of gene-specific primers was designed, following the instructions from the manufacturer of the kit. These primers were verified using OligoAnalyzer 3.1 software (IDT) to amplify a short fragment from each gene (Table 3). *Act2*, a reference gene, was used as the calibrator for the normalization.<sup>34</sup>

In order to optimize various parameters, such as the template concentration and the primer concentration to achieve a reliable comparison among the samples and the controls, calibration curves were constructed for all the genes under study. Using the control sample cDNAs as templates, five or four dilution points, each point representing a 10-fold dilution were plotted against the respective CT values to calculate the efficiency,  $R^2$  and slope. For the quantification, three lines of the transgenic plants (biological replicates) were used and each line was analysed in triplicate (technical replicates). In addition, non-template controls (NTC) were also prepared for each primer

pair. The 15 µL PCR reaction components include; 30 ng of first strand cDNA template (final conc. 2 ng µL<sup>-1</sup>), 7.5 µL Kapa SYBR Fast qPCR Master Mix (2×), 0.125 µM of each primer and nuclease-free water to a total of 15 µL. The PCR program adjusted for the amplification of target fragments, using a Bio-Rad IQ5 optical detection system, consists of: one cycle at 95 °C for 3 min; and 40 cycles of: 95 °C for 10 s, 60 °C for 30 s and 72 °C for 30 s. The amplification curve program was followed by a melting curve program that consisted of 61 cycles, increasing the temperature in each cycle by 0.5 °C and dwelling for 6 s from 65 °C to 95 °C. The PCR amplification of the reference and the target genes from transgenic *A. thaliana* cDNA templates, as well as cDNA templates from the wild-type plants, was performed in separate reactions. Template cDNA from the wild-type plants was used as a control, to calculate the fold change in gene expression levels by determining the threshold cycle ( $C_t$ ) values of the target and the calibrator genes from the control and the test samples using the Livak method.<sup>35</sup> The PCR products were also analysed on 1.2% (w/v) agarose gel electrophoresis to identify the amplification of any non-specific sequences.

### Statistical analysis

Statistical significance was assessed by using GraphPad Prism 5. Data is presented as mean ± SD and the comparison among the three groups was assessed by using one-way ANOVA followed by Dunnett's ultiple comparison test. Differences under  $P < 0.05$  were considered statistically significant.

### Extraction of GLV for SPME-GC-MS analysis

Pre-labelled 20 mL glass vials from Agilent Technologies (USA) were used to collect the samples for this purpose. In each vial, 4 glass beads were added to disrupt the plant tissue for the release of GLV. About 1–2 g of healthy leaves were cut from each pot of plants with a razor blade and quickly transferred to the respective vials. The leaves were further cut into small pieces by the same blade during shifting of plant material to the vial, so as to facilitate the leaves' disruption process. The vials were sealed with parafilm and the crimp-top caps were tightened before weighing the vials.

The GLV emitted from the *Arabidopsis* leaf samples were analysed by extracting these volatiles through the headspace solid-phase micro extraction (HS-SPME) technique and GC-MS, following a recently published protocol from Matsui *et al.* (2012) with some modifications.<sup>36</sup> The tissues were completely disrupted by vigorously vortexing the vials for 5–10 min. Then the vials were incubated at 25 °C for 10 min followed by the addition of 1 mL of saturated solution of calcium chloride to deactivate any enzyme activity.

**Table 3** Reference and gene-specific primers for RT-qPCR

Target gene/primers	Sequence (5'–3')	Amplicon size
Act2-forward	CTTGCACCAAGCAGCATGAA	68
Act2-reverse	CCGATCCAGACACTGTACTTCCTT	
Lox2-forward	CGTGACGCGCAAGTCTTGTCAA	130
Lox2-reverse	CGGGTAAGCCTTCCTGGTCAA	
Hpl-forward	CGCACGTGCGAGGAAAGA	88
Hpl-reverse	CGGCTGATAACCACAAAGAAGCTCA	
Adh-forward	GGAGTGGAGTACAGCTTCGAGTGT	130
Adh-reverse	GGATAGGGAGAAGCTGTGGGCTT	





### SPME-GC-MS analysis

GLV formed by completely disrupted plant material were analyzed by exposing an SPME fiber (50/30 mm DVB/Carboxen/PDMS, Sigma-Aldrich, USA) in the headspace of the vials at 40 °C for 30 min. Subsequently, the fiber was inserted into the insertion port of a GC (Agilent 7890A) containing a 0.25 mm by 30 m BP20 (WAX) column (SGE Analytical Science, Australia) for thermal desorption of the volatiles. The GC was directly coupled to the MS of an Agilent 5975C inert MSD with a triple-axis detector. To completely remove any compound absorbed on the fiber matrix, the fiber was held in the injection port for 10 min. The temperature of the injector was 200 °C and the interface was at 230 °C. The column temperature program followed was: 40 °C for 1 min, rising at a rate of 15 °C per min to 230 °C and then kept there for 1 min. Helium was used as the carrier gas and delivered at a flow rate of 1 mL per min. The analysis was performed by splitless injection with a sampling time of 1 min. The MSD Chemstation was used to find all the peaks in the raw GC chromatogram. A library search was carried out for all the peaks using NIST/EPA/NIH version 2.0, and the results were combined in a single peak table. Among all the GLV, Z-3-hexen-1-ol can serve as the best compound for the analysis of the HPL pathway because it is the first C6 alcohol formed in the pathway. A standard curve was constructed for Z-3-hexen-1-ol consisting of four concentration points at 5, 50, 500 and 1500 nanomolar (nM) concentrations.

## Results and discussion

The physiological role of *AtSKIP11* in the *Arabidopsis* HPL pathway was determined by generating transgenic *A. thaliana* plants, integrating the overexpression and the antisense suppression constructs of the *AtSKIP11* gene. Three of the transgenic lines from each of the *AtSKIP11* overexpressing plants and the *AtSKIP11*-antisense plants were selected for further analysis by RT-qPCR and SPME-GC-MS. Wild-type plants were also grown along with the transgenic plants. Seeds from each of the above-mentioned lines were planted on soil and the plants grown for six weeks under standard conditions. The plants were treated with methyl-jasmonate, to activate the *Arabidopsis* defence-related pathways, including the oxylipin pathway genes, two hours prior to collecting the samples for analysis.<sup>37</sup> The leaf samples were analyzed to measure any changes in the levels of oxylipin gene expression and the GLV formed in transgenic plants against the control plants.

### RT-qPCR analysis

Total RNA was extracted from 100 mg of the leaf powder by PureLink Plant RNA Reagent (Life Technologies), according to the manufacturer's protocol. cDNA was synthesized with a ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs), according to the manufacturer's protocol, using 500 ng of purified total RNA as the template. The cDNA product was diluted with nuclease-free water to a final concentration of 2 ng  $\mu\text{L}^{-1}$ , based on the concentration of the RNA.

The cDNAs of the *AtSKIP11* overexpressing and the *AtSKIP11*-antisense sequence expressing transgenic lines were used as templates to determine any change in the gene expression levels by RT-qPCR, using a pair of gene-specific primers for the target genes and the calibrator gene (reference gene). The relative expression of the genes of the HPL pathway was calculated by averaging the threshold cycle (CT) values of each line and the fold changes in the expression levels of the target genes were calculated by applying the  $2^{-\Delta\Delta C_t}$  method (Appendix A, ESI†). Changes in the gene expression levels for the transgenic plant cDNAs were plotted on a graph to highlight the differences (Fig. 1).

Reduction in the expression of *LOX2*, *HPL* and *ADH1* was shown by the *AtSKIP11* overexpressing plant cDNA templates. The highest change was observed in the expression of *ADH1*, in which more than 2-fold down-regulation was recorded as compared with the control samples. On the other hand, the plants expressing the *AtSKIP11*-antisense sequence gave a different pattern of expression. In these cDNA samples, *LOX2* was down-regulated by about 1.2-fold, while the *HPL* expression was increased by the same value and the *ADH1* was up-regulated by more than 1.7-fold as compared to control plants. The data assessed by using one-way ANOVA followed by Dunnett's multiple comparison Test shows that the difference in expression levels of *ADH1* is statistically significant with a *p*-value of 0.0013.

### SPME-GC-MS analysis of GLV

Products of the HPL pathway were analyzed by GC-MS after extracting the volatiles, produced from the transgenic *A. thaliana* plants integrating the *AtSKIP11* gene and the *AtSKIP11*-antisense sequence by HS-SPME. The GC-MS chromatograms yielded well-resolved peaks for different volatiles with similar patterns (Appendix B, ESI†).

Amounts of different GLV produced by each line of transgenic *A. thaliana* plants and the wild-type *A. thaliana* plants were

Relative Gene Expression in *AtSKIP11* overexpressing and *AtSKIP11*-antisense Plants

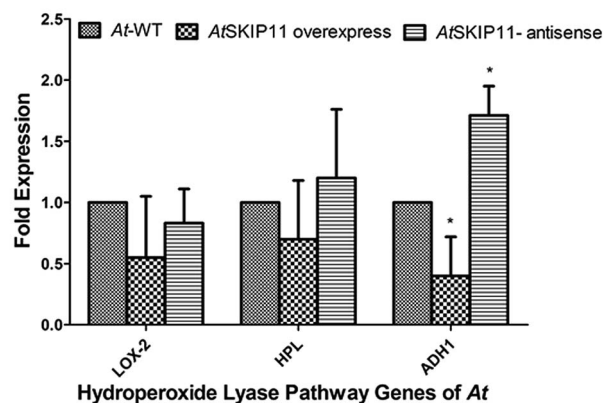


Fig. 1 Changes in the expression levels of HPL pathway genes in the transgenic plants overexpressing the *AtSKIP11* gene and the *AtSKIP11*-antisense sequence with respect to the wild-type plants. Significance is represented by the symbol \*, representing a *P*-value < 0.05.



calculated by averaging the mass abundance of each compound per gram of fresh leaf material from the respective chromatograms and the RSD was determined (Appendix B, ESI†). Average mass abundance values of each volatile compound for the transgenic and control plants are computed in Table 4 along with their retention times.

The amounts of different GLV per gram of fresh leaf material and their RSD values were plotted on a graph, to compare the levels of volatiles produced in the transgenic plants and the wild-type plants (Fig. 2 and 3).

The synthesis of all target GLV compounds was found to be greatly reduced in the *AtSKIP11* overexpressing plants as compared to the control plants, with the highest reduction observed in the formation of two C5 GLV, (*E*)-2-pentenal and 1-penten-3-ol (Fig. 2). The *AtSKIP11*-antisense sequence expressing plants also produced lower amounts of some of the volatile compounds, except (*Z*)-3-hexen-1-ol and 1-hexanol that were either produced in higher concentrations in the transgenic plants or remained almost the same in both types of plants, respectively (Fig. 3). The amounts of (*Z*)-3-hexen-1-ol released by the *AtSKIP11* overexpressing plants was about 55% less, but that released by the *AtSKIP11*-antisense sequence expressing plants was about 20% higher than that produced by the control plants. Concentrations of (*Z*)-3-hexen-1-ol, the key GLV in this study, released by the *AtSKIP11* overexpressing and the *AtSKIP11*-antisense expressing transgenic plants were calculated to be 89 nM and 313 nM per g of fresh leaf, respectively. Shiojiri *et al.* (2012) reported that the maximum concentration of (*Z*)-3-hexenal, (*E*)-2-hexenal, and (*Z*)-3-hexenol released by completely damaged tissue was calculated to be 496 nM GLV per g of the fresh weight.<sup>38</sup>

The reduction in the levels of C5 volatiles to a very significant degree show that the gene may be affecting *LOX1* more than *LOX2* in the *Arabidopsis*. The genes involved in the biosynthesis of C5 volatiles have not been identified until now and, moreover, synthesis of C5 volatiles is found to be independent of HPL.<sup>39</sup> Shen *et al.* (2014) have elucidated the role of TomloxC, a tomato 13-LOX in the biosynthesis of C5 volatiles.<sup>40</sup> *Arabidopsis* LOX1 catalyses the oxygenation at carbon number 9 of the hydrocarbon backbone in LA and ALA to yield (9*S*,10*E*,12*Z*)-9-hydroperoxyoctadeca-10,12-dienoic acid (9-HPOD) and (9*S*,10*E*,12*Z*,15*Z*)-9-hydroperoxy-10,12,15-octadecatrienoic acid (9-HPOT), respectively.<sup>41</sup> The *AtSKIP11* protein has five kelch-repeats and interacts with the substrate proteins possibly through the blades of its  $\beta$ -propeller, thus forming a SCF<sup>At2g02870</sup> (SCF<sup>*AtSKIP11*</sup>) complex.<sup>29</sup>

Analysis of C5 Volatiles in *AtSKIP11* Over-expressing & antisense Plants

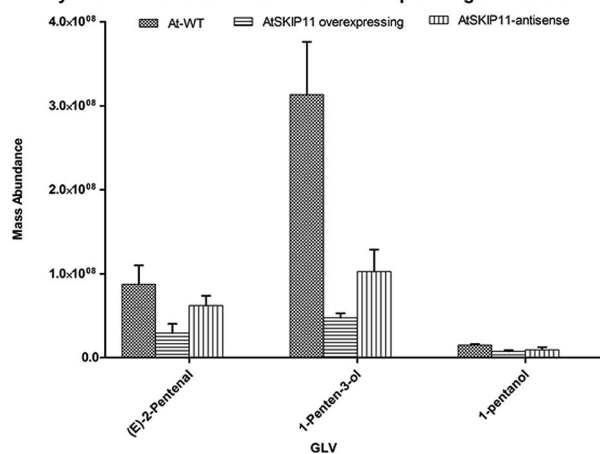


Fig. 2 Relative mass abundance of different C5 volatiles per gram of fresh leaf material in transgenic plants and wild-type plants.

GLV Analysis in *AtSKIP11* Over-expressing & antisense Plants

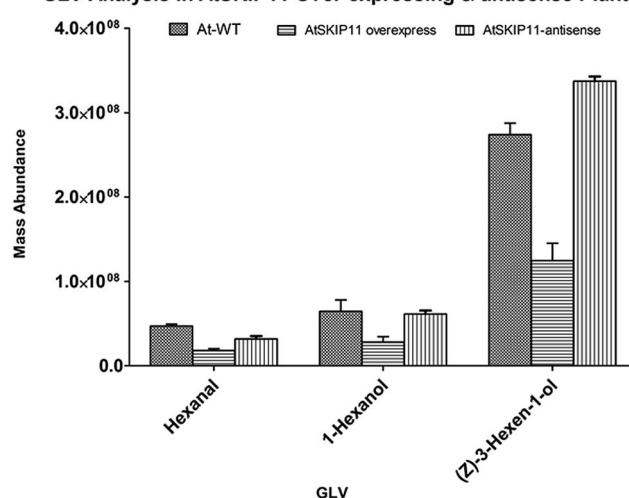


Fig. 3 Relative mass abundance of different GLV per gram of fresh leaf material in transgenic plants and wild-type plants.

The overall picture obtained from the results of these transgenic plants has highlighted the involvement of *AtSKIP11* in the HPL branch of the oxylipin pathway. The protein is suggested to up-regulate a certain portion of the pathway through degradation of some transcription factors or other regulatory

**Table 4** Retention times and the abundance of different volatile compounds in the control plants and the transgenic plants overexpressing the *AtSKIP11* gene and *AtSKIP11*-antisense sequence, obtained by averaging the data from GC-MS chromatograms

GLV	RT (min)	At-WT plants	<i>AtSKIP11</i> overexpress	<i>AtSKIP11</i> antisense
Hexanal	4.80	47 282 321	18 185 543	31 974 550
( <i>E</i> )-2-pentenal	6.27	87 611 380	29 430 781	62 141 721
1-Penten-3-ol	7.60	313 233 340	47 704 923	102 645 089
1-Pentanol	10.36	14 968 059	7 383 709	9 437 418
1-Hexanol	13.42	64 605 639	28 187 494	61 574 701
( <i>Z</i> )-3-Hexen-1-ol	14.27	274 028 143	124 994 804	337 579 587



proteins. Overall, these results suggest that *AtSKIP11* is involved at some stage, most probably downstream of the HPL enzyme, of the HPL biosynthetic pathway in a 'negatively regulating' manner. This means that higher the levels of *AtSKIP11*, the lower will be the production of GLV and *vice versa*. Recently, a few KFBs have been reported to participate in plant physiological phenomena by degrading the inhibitors of the pathway. In a recent report, a novel KFB encoding gene from rice, an inhibitor for brown furrows1 (*IBF1*) was cloned to show that *IBF1* is involved, as a negative regulator in the biosynthesis of flavonoids and to reduce the accumulation of brown pigments in rice hulls.<sup>42</sup> Similarly, a KFB encoding gene in rice, LARGER PANICLE (LP) was found to be involved in the regulation of panicle architecture. This study reported that LP is an endoplasmic reticulum localized protein and possibly modulates the cytokinin levels by interacting with rice SKP1-like protein. Moreover, the mutant plants were found to have increased panicle size, better plant architecture and improved grain yield per plant.<sup>43</sup> The role of the KMD family of proteins was recently reported in the regulation of *Arabidopsis* cytokinin signalling. In the SCF<sup>KMD</sup> complex, KMD proteins were suggested to physically interact with some key transcription factors in the cytokinin signalling. *KMD* loss of function mutants were more sensitive to cytokinin, while transgenic lines overexpressing *KMD* were insensitive to cytokinin, showing that *KMD* proteins negatively regulate the cytokinin signalling in *Arabidopsis*.<sup>44</sup> Recently, a couple of similar studies have highlighted the role of a family of four similar KFBs in *Arabidopsis* in the regulation of the phenylpropanoid biosynthetic pathway. Zhang *et al.* (2013 and 2014) have shown that these proteins regulate the biosynthesis of phenylpropanoids by interaction with and proteolytic turn-over of four phenylalanine ammonia-lyase (PAL) isozymes that catalyse the first and the committed step in phenylpropanoid biosynthesis.<sup>45,46</sup>

Using the results of present study in the context of these published results, a mechanism for the function of *AtSKIP11* is proposed here. *AtSKIP11* interacts with SKP1 through its N-terminal F-box domain and interacts with the target proteins, like LOX2, HPL, ADH1 or the regulatory elements of these genes, through its C-terminal domain. The C-terminal of *AtSKIP11* consists of a five kelch-repeats containing domain, which is thought to form a bladed  $\beta$ -propeller. Each blade of the  $\beta$ -propeller consists of a single kelch-repeat that is folded into four sheets and may be capable of independently interacting with other different proteins.<sup>25</sup> Therefore, it is suggested that multiple interacting partners may exist for the SCF<sup>*AtSKIP11*</sup> complex. The results of the present study show that some proteins, including the enzymes of the oxylipin pathway do interact with the SCF<sup>*AtSKIP11*</sup> complex. Degradation of these proteins, that may be enzymes themselves or regulatory proteins, such as transcription factors, reduce the formation and release of certain GLV, as shown by the transgenic plants overexpressing the *AtSKIP11* gene. The levels of various GLV were found to be reduced in these plants and the expression of the HPL pathway genes was also found to be decreased as compared with the control plants (Fig. 1–3). On the other hand, in the transgenic plants expressing the antisense suppression

construct, levels of some GLV were found to be enhanced and also the expression of the *LOX2* and *HPL* genes was slightly up-regulated, while the expression of *ADH1* was significantly up-regulated (Fig. 1–3). That means that the suppression of the translation of the *AtSKIP11* gene transcript leads to formation of a reduced amount of SCF<sup>*AtSKIP11*</sup> complex. This is in accordance with the results of some recent studies, in which certain KFB overexpressing plants resulted in the down-regulation of the metabolic pathways under study while the mutant plants came up with up-regulation of the pathways.<sup>24</sup> As a result, the degradation of the protein targets for the SCF<sup>*AtSKIP11*</sup> complex will be decreased. The reduced or the retarded protein degradation, of either the enzymes or the transcription factors, will allow the production of more GLV and the up-regulation of certain enzymes of the pathway. According to our proposed mechanism, the impaired or lower expression of *AtSKIP11* leads to decreased production of SCF<sup>*AtSKIP11*</sup> complex and thus less degradation of GLV-producing proteins.

It is obvious from the literature review of the GLV biosynthetic pathway that the pathway is quite complex. Release of PUFAs by the degradation of membrane lipids is still not completely understood. LA and ALA, released by phospholipases, serve as substrates for the oxylipin pathway, but there may exist some other PUFA candidates as well. Although *LOX2* contributes more towards the generation of oxygenated intermediates, the role of the regulatory components switching between the two types of *LOX* may also contribute significantly. The most important step in the pathway is the distribution of oxygenated substrates between the HPL and the AOS branches of the pathway. The importance of jasmonic acid is not only to rescue the plant during any type of stress, but it is involved in almost every aspect of plant growth and development.<sup>9</sup> While only trace amounts of GLV are released by plants under normal physiological conditions, they are formed very rapidly under stressful conditions. Numerous studies have suggested that the two competing branches of the oxylipin pathway crosstalk during metabolism and the stress response.<sup>14</sup> The role of the regulatory proteins at this stage of the pathway is of great importance, in which SCF complexes may be very crucial. The degradation of certain proteins will allow the pathway to switch between the two branches of the pathway. Among the downstream genes, *ADH1* appears to be more responsive towards the KFB of the present study. Either *ADH1* itself or the transcription factors that enhance its expression seem to be degraded by over-expressed *AtSKIP11* (Appendix C, ESI†).

## Conclusions and future prospects

The results of the analysis of transgenic plants manipulating the *AtSKIP11* gene, which is endogenous to *Arabidopsis* and its antisense suppression sequence, suggest a role for the KFB *AtSKIP11* in the *Arabidopsis* HPL branch of the oxylipin pathway. The release of a higher concentration of GLV under stressed conditions led us to conclude that gene expression for the GLV biosynthetic pathway is induced by signals from infection or stress. Therefore, the nature of stress signals and the mechanisms by which these signals give rise to the generation of the



defence response remain elusive. Although many studies have shown encouraging results, most are limited to laboratory experiments that were performed using synthetic GLV.<sup>14</sup> It is the need of the hour to devise new strategies and to design novel substrates for identifying the 'missing link' between the perception of a stress signal and the generation of a defence response. The proposed mechanism from our results suggests that the reduced expression of *AtSKIP11* leads to decreased production of the SCF<sup>AtSKIP11</sup> complex and thus less degradation of GLV-producing proteins and *vice versa*. So, *AtSKIP11* may be one of the 'missing links' in the *Arabidopsis thaliana* HPL pathway and may serve as a molecular switch for controlling the production of GLV.

## Conflicts of interest

The authors declare no conflicts of interest.

## Abbreviations

ALA	$\alpha$ -Linolenic acid.
DES	Divinyl ether synthase.
DGL	DONGLE.
GLV	Green leaf volatiles.
HIPVs	Herbivore-induced plant volatiles.
13-HOT	13-Hydroxytridecanoic acid.
13-	(9Z,11E,13S)-13-Hydroperoxyoctadeca-9,11-dienoic acid
HPOD	acid
13-	(9Z,11E,15Z)-13-Hydroperoxyoctadeca-9,11,15-
HPOT	trienoic acid.
JA	Jasmonic acid.
LA	Linoleic acid.
PUFAs	Polyunsaturated fatty acids
KFBs	Kelch-repeat containing F-box proteins
SPME	Solid phase micro-extraction.
MeJA	Methyl jasmonate.

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