Modulation of GmFAD3 expression alters responses to abiotic stress in soybean

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Abstract

FAD3 play important roles in modulating membrane fluidity in response to various abiotic stresses. However, a comprehensive analysis of FAD3 in drought, salinity and heat stress tolerance is lacking in soybean. The present study assessed the functional role of fatty acid desaturase 3 to abiotic stress responses in soybean. We used Bean Pod Mottle Virus -based vector to alter expression of Glycine max omega-3 fatty acid desaturase . Higher levels of recombinant BPMV-GmFAD3 transcripts were detected in overexpressing soybean plants. Overexpression of GmFAD3 in soybean resulted in increased levels of jasmonic acid and higher expression of GmWRKY54 as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants under drought and salinity stress conditions. FAD3 overexpressing plants showed higher levels of chlorophyll content, leaf SPAD value, relative water content, chlorophyll fluorescence, transpiration rate, carbon assimilation rate, proline content and also cooler canopy under drought and salinity stress conditions as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants. Results from current study revealed that GmFAD3 overexpressing soybean plants exhibited drought and salinity stress tolerance although tolerance to heat stress was reduced. On the other hand, soybean plants silenced for GmFAD3 exhibited tolerance to heat stress, but were vulnerable to drought and salinity stress

1 INTRODUCTION

Soybean [*Glycine max* (L.) Merr] is major oilseed crop in the world and an important source of protein and oil for both humans and animals. It is also used as a raw product for many human health and industrial applications. Therefore, demand for soybean is increasing continuously worldwide. Plant growth, development and yield potential are greatly affected by abiotic stresses such as drought, salinity and low/high temperature (Polizel *et al* ., 2011). Abiotic stresses like drought, salinity, extreme ambient temperatures, and freezing cause severe losses to soybean productivity worldwide by adversely affecting plant growth, development and yield potential. Thus, understanding abiotic stress responses in soybean and enhancing abiotic stress resilience to maintain genetic yield potential are extremely demanding areas in agricultural research. This requires improvement in the tolerance of soybean plants to environmental stresses.

Fatty acids are vital cellular constituents of plants since they contribute to cellular membrane architecture, suberin and cuticular waxes as well as to meeting the energy requirements of cells. Fatty acids also play a key role in signal transduction (Wang., 2004). Predominant polyunsaturated fatty acids found in most plant seed oils are linoleic acid and α-linolenic acid (Napier and Graham., 2010). The role of free linolenic acid as

a stress signal and precursor of oxylipin and jasmonic acid biosynthesis in plants has also been emphasized (Upchurch., 2008; Martin *et al.* , 2018). Jasmonic acid is synthesized from 12-OPDA with different metabolic conversions and physiological processes and triggers defense response as well as responses to abiotic stresses (Supplementary Fig. S1).

Fatty acids present in commodity soybean oil are palmitic acid (10%), stearic acid (4%), oleic acid (18%), linoleic acid (55%) and linolenic acid (13%). However, higher level of linolenic acid limits the stability of soybean oil to oxidation (Clemente & Cahoon., 2009). Moreover, the thermal instability of these fatty acids also renders oil unsuitable for other uses like engine lubricants or hydraulic fluids. Owing to these reasons, crop breeding emphasizes oilseed crops with low polyunsaturated fatty acid contents. Genes controlling contents of oleic acid and polyunsaturated fatty acids have been identified in soybean (Pham *et al* ., 2012). Fatty acid desaturase 3 (FAD3) enzymes catalyze conversion of linoleic acid to α-linolenic acid includes three active members viz. FAD3A, FAD3B and FAD3C. However, FAD3A has been reported to have a higher expression in seeds compared to FAD3B and FAD3C. FAD3 mediated unsaturation takes place in Endoplasmic Reticulum (ER) where phosphatidylcholine is the primary lipid substrate (Chapman and Burke., 2012).

Overexpression of *FAD3* or *FAD8* increased tolerance to osmotic stress in tobacco plants although thermal tolerance was reduced (Zhang *et al* ., 2005). In another study, tobacco plants silenced for *FAD7* showed decreased levels of linolenic acid and exhibited reduced drought and salinity stress tolerance (Im *et al* ., 2002). Arabidopsis plants double mutant for*fad7 /fad8* , deficient in linolenic acid, exhibited susceptible response to cold and conferred tolerance to elevated temperature (Zhang *et al* ., 2005). Overexpression of fatty acid desaturases (FAD3 and FAD7) enhanced tolerance to cold stress in tomato (Dominguez *et al* ., 2010). In recent years, some novel genes/transcription factors have been characterized for modulating stress responsive genes associated with abiotic stress tolerance by genome-wide characterization (Li *et al* ., 2019; Do *et al* , 2019). Efforts have also been made to enhance abiotic stress tolerance in soybean by manipulating expression of several genes/transcription factors (Jumrani & Bhatia., 2019; Zhang*et al* ., 2019; Chen *et al.* , 2019). Flores *et al.*(2008) silenced *FAD3* gene employing siRNA-mediated approach in soybean, however comprehensive analysis of their roles in drought and salinity stress responses was not carried out.

In the present investigation, we assessed the functional roles of FAD3 to abiotic stress responses in soybean and it was observed that the*FAD3* overexpressing plants (OE-FAD3) exhibited tolerance to drought and salinity stresses, although tolerance to heat stress was reduced. On the other hand, soybean plants silenced for *GmFAD3*exhibited tolerance to heat stress, but were found vulnerable to drought and salinity stress. Hence, soybean with higher linolenic acid content could be desirable plant types with regard to drought and salinity stress tolerance, while soybean with reduced level of linolenic acid could be useful for thermal tolerance.

2 MATERIALS AND METHODS

2.1 Soybean cultivars and plant growth conditions

Soybean cvs. Essex, Harosoy, Williams, NRC-37, and JS-335 were grown in a greenhouse with day and night temperatures of 27 and 24°C, respectively. Carborundum-dusted soybean plants at VC growth stage with unifoliate leaves were rub-inoculated with different treatments. Soybean plants at the V3 growth stage, which were previously inoculated on the unifoliate leaves with mock-inoculated, vector-infected, S-FAD3 and OE-FAD3 plants, were used in all experiments. All experiments were repeated at least three times. All plants used for testing responses to drought, salt concentrations and heat stress were verified for*GmFAD3* mRNA content by semi-quantitative RT-PCR and also RT-qPCR prior to withholding watering, salt treatment and elevated temperature at 42°C.

2.2 Construction of viral vectors, *in vitro* transcription and plant inoculation

For BPMV-based overexpression of *GmFAD3* , a full-length cDNA (1125 bp) of coding sequence of *GmFAD3* gene was amplified by RT-PCR from soybean cDNA using primers

(forward primer- 5'-AAAACGCCTATGGTTAAAGACACAAAG-3' and reverse primer- 5'-AAAAGGCCTGTGTCGTTGCGAGTGGAG-3'). Primers were designed for full-length coding sequences available in the database of *GmFAD3*(AY204710). The PCR product was digested with *Stu* I and cloned into *Msc* I digested pGG7R2V-BPMV vector. Construction of silencing vector used in the present study was described previously (Singh*et al* ., 2011). *In vitro* transcription and rub-inoculation of soybean plants were carried out as described before (Zhang and Ghabrial., 2006; Diaz-Camino *et al* ., 2011; Singh *et al* ., 2011; Kachroo & Ghabrial., 2012; Rao *et al* . 2014; Shine *et al* ., 2016). Freeze-dried transcript-infected leaves were used to prepare inoculum for the various treatments. The mock-inoculated, vector-infected, S-FAD3 and OE-FAD3 soybean plants were used for molecular analysis and for evaluating tolerance to drought, salinity and elevated temperature stresses, 21 days post-infection.

2.3 RNA extraction, Reverse transcriptase PCR, quantitative Real-Time PCR and RNA blot analyses

Total RNA was extracted from leaf tissues of mock-inoculated, vector-infected, OE-FAD3 and S-FAD3A plants, 21 days post-infection, using RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. First-strand cDNA synthesis was performed using SuperScript® II Reverse Transcriptase (Invitrogen). To evaluate relative differences in FAD3 transcript levels in mock-inoculated, vector-infected, OE-FAD3 and S-FAD3 soybean plants, three independent RNA preparations were analyzed by semi-quantitative Reverse Transcriptase-mediated PCR (RT-PCR) involving 25 cycles using forward primer- 5'-AAAACGCCTATGGTTAAAGACACAAAG-3' and reverse primer- 5'-AAAAGGCCTGTGTCGTTGCGAGTGGAG-3' to amplify a PCR product of 1125 bp. To assess the expression of *GmWRKY54* transcription factor at transcript level in mock-inoculated, vector-infected, S-FAD3 and OE-FAD3 plants under well-watered, drought and salinity stress conditions, semi-quantitative RT-PCR was performed. RT-PCR for *β-Τυβυλιν*transcripts from mock, vector-infected, S-FAD3 and OE-FAD3 plants was also performed using forward primer 5'-CAATTGGAGCGCATCAATG-3' and reverse primer 5'- ATACACTCATCAGCATTCTC-3' to check equal quantity loading of cDNA. The amplified products were analyzed by electrophoresis on a 1% agarose gel. Relative differences in transcript levels in S-FAD3, OE-FAD3A, vector-infected and mock-inoculated soybean plants were also evaluated by quantitative RT-PCR (RT-qPCR). For quantitative Real-Time PCR, cDNA was synthesized and DNA amplification was performed in the presence of SYBR Green Real-Time Bio-Rad PCR master mix on the Bio-Rad CFX 96 Touch Real-Time PCR detection system using primer pairs as listed in Table 1. The relative mRNA levels of FAD3 were determined by normalizing the PCR threshold cycle number with that of*β-Τυβυλιν* . All experiments were repeated three times independently and the average was calculated.

For RNA blot hybridization, total RNA was electrophoresed on a 1% (w/v) agarose gel that contained 2% (w/v) formaldehyde. Subsequently, RNA was transferred onto a zeta probe membrane (Bio-Rad, USA) and then hybridized with a [^{32}P]-dCTP-labeled probe (*GmFAD3* gene and BPMV RNA2-coat protein-specific probe) for 24 h in a phosphate-buffered solution (0.2 M sodium phosphate buffer, 0.25 M sodium chloride, 1 mM EDTA, 7% sodium dodecyl sulphate). Then, the membrane was exposed in a Phosphorimager cassette (Molecular Dynamics, Synnyvale, CA, USA). The intensities of bands were detected using the Image-Quant software (Molecular Dynamics).

2.4 Assessment of tolerance to drought, salinity and heat stress in OE-FAD3, S-FAD3 soybean plants

Ten to 12 individual soybean plants (cvs Essex, Harosoy, Williams, NRC-37 and JS-335) were tested per experiment. To assess the tolerance to drought stress at whole plant level, mock-inoculated, vector-infected, OE-FAD3, and S-FAD3 plants were uniformly subjected to water withholding for 3 days under greenhouse conditions. For salt stress tolerance assessment, plants were treated with 100 and 150 mM NaCl for four days. Similarly, tolerance of whole plants to elevated temperatures was assessed by incubating plants at 42^{o}C for two days in temperature controlled walk-in growth chamber.

2.5 Chlorophyll assay, leaf SPAD value, chlorophyll fluorescence and relative water content

(RWC)

For chlorophyll content estimation, 250 mg ground fresh leaf samples were extracted with 10 ml of DMSO in a test tube and were incubated at 60°C for 6 hours. Absorbance was measured at 665 nm and 649 nm using UV–VIS spectrophotometer (UV-1800, Shimadzu, Japan) and total chlorophyll contents were estimated as described by Barnes *et al* . (1992). The SPAD meter (SPAD-502, Konica Minolta Optics, Inc. Japan) was used to measure the greenness or relative chlorophyll content of leaves. The V3 stage soybean plants of mock-inoculated, vector-infected, S-FAD3, OE-FAD3 plants under well-watered, drought stress and salinity stress conditions (100 and 150 mM NaCl) were used for SPAD measurement. A mean of 3 values per plant was taken for measuring SPAD value. A total 9 plants were analyzed for SPAD value. Chlorophyll fluorescence was measured in excised leaves at V3 stage of soybean plants 21 days post-infection. The leaf images were captured by chlorophyll fluorescence measuring system (PSI, Czechoslovakia) and analysis of data was performed using fluorochrom7 software. The RWC was determined 21 days post-infection at V3 stage. It was calculated using following equation, RWC = (FW-DW) X 100 / (SW-DW), where FW is the fresh weight, SW is the water-saturated weight and DW is the dry weight (Turner, 1981). Trifoliate leaves were excised from plants and were weighed immediately to record fresh weight, then turgid weight was determined by soaking leaves in water for 6 h in distilled water at room temperature and then surface water was removed and leaves were weighed. Dry weight was measured after drying the leaves in oven at 65°C for 72 hours.

2.6 Canopy temperature, Leaf stomatal conductance, transpiration and CO_2 assimilation rate

The Canopy Temperature Depression (CTD) was measured with a handheld thermometer, IR-Gun at V3 stage of soybean plants 21 days post infection. The data were taken approximately 50 cm above the canopy. Stomatal conductance of leaves was determined using Porometer (Delta-T AP4, Delta-T Devices, Cambridge, UK). Third trifoliate leaves of 9 replicates were taken for measurement. The Porometer was calibrated before taking measurement. Transpiration rate and CO_2assimilation rate were measured on trifoliate leaves of plants with a portable gas exchange fluorescence system (GFS 3000, WALZ) at a photosynthetic photon flux density ranging from 900 to 1200 μmol m^{-2} s^{-1}, with air temperature of 23 ± 1°C) to 28 ± 2°C.

2.7 Proline content

Free proline content was estimated using the ninhydrin method described by Bates *et al.* (1973). Fresh leaf samples (500 mg) were used for proline extraction. Optical density was measured at 520 nm using toluene as blank using UV–VIS spectrophotometer (UV-1800, Shimadzu, Japan). The amount of proline was determined from a standard curve.

2.8 Jasmonic acid analysis

For jasmonic acid extraction, 1 gram of leaf tissue was ground in cold 100% methanol with dihydrojasmonic acid as an internal standard. The methanol extract was passed through Seppak-18 column (Waters: Sep-Pak Classic C18 cartridge). The column purified extract was processed as described by Xia *et al* . (2009) and injected into gas chromatograph attached to an electron ionization detector (Hewlett Packard GCD Systems). The JA peaks were identified using mass spectrometry.

2.9 Statistical analysis

Data obtained from biochemical (Proline content, JA level), physiological analysis (chlorophyll content, SPAD value, Relative Water Content, chlorophyll fluorescence, stomatal conductance, transpiration rate, CO_2 assimilation rate and canopy temperature), and RT-qPCR were subjected to analysis of variance (ANOVA). Values were represented as mean ± standard errors. Each experiment was repeated three times. Significant differences among the mean values were compared using the Student's t Test.

3 RESULTS

3.1 Higher accumulation of BPMV-RNA2-GmFAD3 recombinant transcripts in *GmFAD3*-overexpressing plants

For overexpression of *GmFAD3* , a full length cDNA (1125 bp) containing the complete coding sequence of *GmFAD3* , excluding the termination codon, was inserted into the *Msc* I site of the BPMV vector. To assay the accumulation of endogenous *GmFAD3* or recombinant BPMV-RNA2:GmFAD3 transcripts, semi-quantitative RT-PCR analysis was performed using primers designed to amplify the full length coding sequence of GmFAD3. Densitometry analysis revealed about 5 times higher accumulation of *GmFAD3* transcripts in overexpressing plants compared to mock-inoculated and vector-infected soybean plants (Figure 1a). The RT-qPCR was also conducted to analyze endogenous *GmFAD3* or recombinant *BPMV-RNA2:GmFAD3* transcripts. The RT-qPCR analysis revealed about three-fold decrease in mRNA levels in S-FAD3 (FAD3-silenced) soybean plants, while about five-fold higher mRNA levels in OE-FAD3 (FAD3 overexpressing) soybean plants (Figure 1b). Soybean plants infected with recombinant vector carrying a full-length cDNA of *GmFAD3* showed distinct phenotype as compared to the vector-infected and FAD3-silenced (S-FAD3) soybean plants (Figure 1c). To further validate the transcript abundance for *GmFAD3* , Northern hybridization blots were probed using *GmFAD3* specific probe (Figure 1d). *GmFAD3* overexpressing (OE-FAD3) plants revealed two bands corresponding to endogenous *GmFAD3* transcripts and recombinant *BPMV-RNA2-GmFAD3* transcripts. In mock-inoculated and vector-infected, as expected, a single RNA band corresponding to endogenous *GmFAD3* was detected. Due to the strong hybridization signals from recombinant *BPMV-RNA2:GmFAD3* , the exposure time for overexpression treatments was kept very short ($<$ 30 minutes). Because of differential exposure time, lower abundance for endogenous *GmFAD3* was detected in OE-FAD3 plants when compared to mock-inoculated and vector-infected soybean plants (Figure 1d). Otherwise, levels of endogenous *GmFAD3* were similar in mock-inoculated, vector-infected and *FAD3* overexpression treatments.

3.2 Overexpression of *GmFAD3* does not alter soybean seed size

Our earlier work (Singh *et al* ., 2011) had demonstrated increase in soybean seed size and weight in *FAD3* -silenced plants as compared to mock-inoculated and vector-infected plants. To study the impact of *GmFAD3* overexpression, these seed traits were analyzed in mock-inoculated, vector-infected and OE-FAD3 plants of soybean cvs. Essex, Harosoy, Williams, NRC-37, and JS-335. Although the *GmFAD3*overexpressing plants exhibited BPMV related symptoms, they produced pods with seeds that were similar to those of vector-infected plants in size and weight.

3.3 Enhanced tolerance to drought and salinity stress in plants with higher *FAD3* expression

Overexpression of *FAD3* or *FAD8* has been reported to increase osmotic stress tolerance in tobacco plants although thermal tolerance was reduced (Zhang *et al* ., 2005). This prompted us to investigate the effect of *GmFAD3* overexpression on drought and salt tolerance in soybean plants. To explore the role of *GmFAD3*in conferring tolerance to drought and salinity stress, *GmFAD3*silenced and the overexpressing plants together with mock-inoculated and vector-infected plants were subjected to water deficit and salinity stress. With increasing water deficit, mock-inoculated, vector–infected and S-FAD3 plants started wilting followed by drooping of leaves, however no leaf drooping was observed in OE-FAD3 plants when subjected to water deficit conditions (Figure 2a). In salinity stress tolerance assay, the GmFAD3 overexpressing plants, GmFAD3 silenced plants along with mock-inoculated and vector-infected soybean plants were subjected to salt stress of 100 and 150 mM NaCl solution in pots filled with soilrite (mixture of peat moss, perlite and vermiculite). Within four days of salt stress, mock-inoculated, vector-infected and S-FAD3 plants exhibited leaf scorching and this scorching progressively led to leaf necrosis with increasing exposure to salt stress. Interestingly, OE-FAD3 plants did not develop any leaf scorching (Figure 2b).

3.4 Enhanced elevated thermal tolerance in FAD3-silenced plants

Thermal tolerance was assessed by exposing mock-inoculated, vector-infected, FAD3 overexpressing and FAD3 silenced plants at 42^{o}C for 2 days in temperature controlled walk-in plant growth chambers. The GmFAD3-silenced plants exhibited thermal-tolerance and did not reveal any marked morpho-physiological damage, while mock-inoculated, vector-infected and FAD3 overexpressing plants were found vulnerable to thermal stress and exhibited turgor loss and drooping symptoms (Figure 2c).

3.5 Higher chlorophyll content, leaf SPAD value, chlorophyll fluorescence and relative water content under drought and salinity stress conditions in FAD3 overexpressing soybean plants

Chlorophyll content was significantly higher in OE-FAD3 soybean plants as compared to mock-inoculated, vector-infected and S-FAD3 soybean plants under drought and salinity stress conditions (150 mM NaCl) (Figure 3a). Interestingly, under well-watered condition, S-FAD3 soybean plants showed higher chlorophyll content as compared to mock-inoculated, vector-infected plants (Figure 3a). In the present investigation, it was observed that decrease in chlorophyll content was less in OE-FAD3 plants as compared to mock-inoculated, vector-infected and S-FAD3 under drought and salinity stress condition. When mock-inoculated, vector-infected, and S-FAD3 plants were treated with 150 mM of NaCl for 4 days, chlorophyll content decreased from 2.13 ± 0.14 mg/g FW to 0.99 ± 0.07 mg/g FW, 1.86 ± 0.15 mg/g FW to 0.76 ± 0.07 mg/g FW, 2.84 ± 0.26 to 1.10 ± 0.09 mg/g FW, respectively. In contrast, when OE-FAD3 plants were subjected to 150 mM NaCl, the chlorophyll content decreased from 2.54 ± 0.25 mg/g FW to 2.03 ± mg/g FW. During exposure to drought stress due to withholding watering for 3 days, the chlorophyll content decreased from 2.13 ± 0.14 mg/g FW to 1.37 ± 0.12 mg/g FW, 1.86 ± 0.15 mg/g FW to 1.24 ± 0.07 mg/g FW, 2.84 ± 0.26 mg/g FW to 1.58 ± 0.11 mg/g FW, respectively in mock-inoculated, vector-infected and FAD3-silenced, but from 2.54 ± 0.25 mg/g FW to 2.32 ± 0.19 mg/g FW in OE-FAD3 plants.

Under well-watered condition, S-FAD3 soybean plants exhibited increase in SPAD value as compared to mock-inoculated, vector-infected plants (Figure 3b). However, under drought and salt stress conditions, OE-FAD3 plants showed significantly higher SPAD value than that of mock-inoculated, vector-infected and S-FAD3 plants (Figure 3b). SPAD value decreased markedly in mock-inoculated, vector-infected, and S-FAD3 plants as compared to OE-FAD3 soybean plants under drought and salinity stress conditions. During exposure to drought stress due to withdrawal of watering for 3 days, the SPAD value decreased from 49.77 ± 3.73 to 38.33 ± 2.44, 45.66 ± 4.02 to 35.22 ± 3.06, 58.88 ± 3.19 to 40.00 ± 2.73, respectively in mock-inoculated, vector infected and S-FAD3 plants, but from 55.36 ± 3.43 to 50.11 ± 3.24 in OE-FAD3 plants. When mock-inoculated, vector-infected and S-FAD3 plants were treated with 150 mM NaCl for 4 days, the SPAD value decreased from 49.77 ± 3.73 to 33.22 ± 3.03, 45.66 ± 4.02 to 31.00 ± 2.30, 58.88 ± 3.19 to 36.55 ± 1.40, respectively. By contrast, when OE-FAD3 plants were subjected to 150 mM NaCl for 4 days, the SPAD value decreased from 55.36 ± 3.43 to 45.44 ± 1.73.

Chlorophyll fluorescence (Fv/Fm) indicates efficiency of Photosystem II which is adversely affected by drought and salinity stress. In the present study, it was observed that value of Fv/Fm was almost similar in mock-inoculated, vector-infected and OE-FAD3 plants while higher value of Fv/Fm was recorded in S-FAD3 soybean plants under well-watered condition (Figure 3c). The chlorophyll fluorescence (Fv/Fm) value decreased markedly in mock-inoculated, vector-infected and S-FAD3 plants when compared to OE-FAD3 plants under drought and salt stress conditions (Figure 3c). When mock-inoculated, vector-infected, and S-FAD3 plants were exposed to salt stress at 150 mM of NaCl for 4 days, the Fv/Fm value decreased from 0.76±0.03 to 0.40 ±0.02, 0.72 ±0.03 to 0.38 ±0.02, 0.85 ±0.02 to 0.48 ±0.02, respectively. In contrast, when OE-FAD3 plants were subjected to 150 mM NaCl, the Fv/Fm value decreased from 0.78 ±0.02 to 0.64 ± 0.03. During exposure to drought stress by withholding watering for 3 days, the Fv/Fm value decreased from 0.76 ±0.03 to 0.45 ±0.03, 0.72 ±0.03 to 0.42 ±0.01, 0.85 ±0.02 to 0.51 ±0.01, respectively in mock-inoculated, vector-infected and S-FAD3 plants, but from 0.78 ±0.02 to 0.69±0.03 mg/g-FW in OE-FAD3 g plants.

Under well-watered, drought and salinity stress condition, OE-FAD3 plants exhibited higher RWC as compared to mock-inoculated, vector-infected and S-FAD3 soybean plants (Figure 3d). It was observed that decrease in RWC was less in OE-FAD3 plants as compared to mock-inoculated, vector-infected and S-FAD3 under drought and salinity stress conditions. During exposure to drought stress due to withdrawal of irrigation for 3 days, the RWC decreased from 65.67 ± 4.20 to 51.89 ± 4.31, 60.11 ± 2.12 to 47.44 ± 2.55, 90.00 ± 3.22 to 60.89 ± 2.18, respectively in mock-inoculated, vector-infected and S-FAD3 plants, but from 85.89 ± 2.08 to 75.22 ± 1.92 in OE-FAD3 plants. When mock-inoculated, vector-infected and S-FAD3 plants were exposed to 150 mM NaCl for 4 days, the RWC decreased from 65.67 ± 4.20 to 45.88 ± 2.54, 60.11 ± 2.12 to

41.44 ± 2.50, 90.00 ± 3.22 to 50.22 ± 2.97, respectively. By contrast, when OE-FAD3 plants were exposed to 150 mM NaCl for 3 days, the RWC decreased from 85.89 ± 2.08 to 65.78 ± 1.96. These results suggested that the overexpression of *FAD3* gene had a strong effect in maintaining the water status in OE-FAD3 plants.

3.6 FAD3 overexpressing plants has lower canopy temperature

OE-FAD3 plants showed lower canopy temperature as compared to mock-inoculated, vector-infected, S-FAD3 plants under well-watered and drought stress conditions (Fig. 4a). Under, drought stress conditions, canopy temperature markedly increased in mock-inoculated, vector-infected and S-FAD3 plants compared to OE-FAD3 plants (Figure 4a). During exposure to drought stress due to withholding watering for 3 days, the canopy temperature increased from 27.78 ± 0.92°C to 35.68 ± 0.82°C, 28.32 ± 0.80°C to 36.33 ± 0.94°C, 26.9 ± 0.84°C to 33.03 ± 0.87°C, 24.01 ± 0.89°C to 26.28 ± 0.94°C, respectively in mock-inoculated, vector-infected, S-FAD3 plants and OE-FAD3 soybean plants. The results showed that under drought stress condition, increase in canopy temperature was lower in OE-FAD3 plants as compared to mock-inoculated, vector-infected and S-FAD3 plants.

3.7 FAD3A overexpressed soybean plants has higher transpiration rate, stomatal conductance, CO_2 assimilation rate

Transpiration controls water absorption from roots and regulates water status of plants. FAD3 overexpressing plants exhibited significantly higher transpiration rate as compared to mock-inoculated, vector-infected plants and S-FAD3 plants under drought and salt stress conditions (Figure 4b). When mock-inoculated, vector-infected, and S-FAD3 plants were subjected to salinity stress at 150 mM of NaCl for 4 days, transpiration rate decreased from 2.02 ± 0.21 mmol $m^{-2}s^{-1}$ to 1.00 ± 0.08 mmol $m^{-2}s^{-1}$, 1.73 ± 0.12 mmol $m^{-2}s^{-1}$ to 0.87 ± 0.06 mmol $m^{-2}s^{-1}$, 2.97 ± 0.23 to 1.56 ± 0.09 mmol $m^{-2}s^{-1}$, respectively. In contrast, when OE-FAD3 plants were subjected to 150 mM NaCl, the transpiration rate decreased from 2.68 ± 0.26 mmol $m^{-2}s^{-1}$ to 2.10 ± 0.12 mmol $m^{-2}s^{-1}$. During exposure to drought stress due to withholding watering for 3 days, the transpiration rate decreased from 2.02 ± 0.21 mmol $m^{-2}s^{-1}$ to 1.43 ± 0.11 mmol $m^{-2}s^{-1}$, 1.73 ± 0.12 mmol $m^{-2}s^{-1}$ to 1.24 ± 0.11 mmol $m^{-2}s^{-1}$, 2.97 ± 0.23 mmol $m^{-2}s^{-1}$ to 1.91 ± 0.13 mmol $m^{-2}s^{-1}$, respectively in mock-inoculated, vector-infected and S-FAD3 plants, but from 2.68 ±0.26 mmol $m^{-2}s^{-1}$ to 2.41 ± 0.18 mmol $m^{-2}s^{-1}$ in OE-FAD3 plants.

Under well-watered conditions, S-FAD3 plants exhibited higher stomatal conductance as compared to mock-inoculated and vector-infected soybean plants. However, OE- FAD3 plants showed significantly higher stomatal conductance as compared to mock-inoculated, vector-infected plants under drought and salt stress conditions (Figure 4c). When mock-inoculated, vector-infected, and S-FAD3 plants were subjected to salinity stress at 150 mM of NaCl for 4 days, stomatal conductance decreased from 0.21 ± 0.01 mmol $m^{-2}s^{-1}$ to 0.12 ± 0.01 mmol $m^{-2}s^{-1}$, 0.18 ± 0.01 mmol $m^{-2}s^{-1}$ to 0.11 ± 0.01 mmol $m^{-2}s^{-1}$, 0.30 ± 0.01 to 0.11 ± 0.01 mmol $m^{-2}s^{-1}$, respectively. In contrast, when OE-FAD3 plants were subjected to 150 mM NaCl, the stomatal conductance decreased from 0.27 ± 0.01 mmol $m^{-2}s^{-1}$ to 0.17 ± 0.01 mmol $m^{-2}s^{-1}$. During exposure to drought stress due to withholding watering for 3 days, the stomatal conductance decreased from 0.21 ± 0.01 mmol $m^{-2}s^{-1}$ to 0.15 ± 0.01 mmol $m^{-2}s^{-1}$, 0.18 ± 0.01 mmol $m^{-2}s^{-1}$ to 0.13 ± 0.01 mmol $m^{-2}s^{-1}$, 0.30 ± 0.01 mmol $m^{-2}s^{-1}$ to 0.16 ± 0.01 mmol $m^{-2}s^{-1}$, respectively in mock-inoculated, vector-infected and S-FAD3 plants, but from 0.27 ± 0.01 mmol $m^{-2}s^{-1}$ to 0.21 ± 0.01 mmol $m^{-2}s^{-1}$ in OE-FAD3 plants.

OE-FAD3 plants showed significantly higher assimilation rate as compared to mock-inoculated, vector-infected plants under drought and salt stress conditions (Figure 4d). When mock-inoculated, vector-infected and S-FAD3 plants were exposed to salinity stress at 150 mM of NaCl for 4 days, assimilation rate decreased from 13.33 ± 1.11 mmol $m^{-2}s^{-1}$ to 7.00 ± 1.00 mmol $m^{-2}s^{-1}$, 10.33 ± 1.20 mmol $m^{-2}s^{-1}$ to 5.66 ± 0.33 mmol $m^{-2}s^{-1}$, 20.11 ± 1.11 to 7.88 ± 0.50 mmol $m^{-2}s^{-1}$, respectively. In contrast, when OE-FAD3 plants were subjected to 150 mM NaCl, the assimilation rate decreased from 17.66 ± 0.93 mmol $m^{-2}s^{-1}$ to 11.50 ± 1.15 mmol $m^{-2}s^{-1}$. During exposure to drought stress due to withholding watering for 3 days, the assimilation rate decreased from 13.33 ± 1.11 mmol $m^{-2}s^{-1}$ to 8.55 ± 0.38 mmol $m^{-2}s^{-1}$, 10.33 ± 1.20 mmol $m^{-2}s^{-1}$ to 7.77 ± 0.38 mmol $m^{-2}s^{-1}$, 20.11 ± 1.11 mmol $m^{-2}s^{-1}$ to 9.0 ± 0.57 mmol $m^{-2}s^{-1}$, respectively in mock-inoculated,

vector-infected and S-FAD3 plants, but from 17.66 ± 0.93 mmol $m^{-2}s^{-1}$ to 13.22 ± 1.01 mmol $m^{-2}s^{-1}$ in OE-FAD3 plants.

3.8 GmFAD3-overexpressing plants accumulate higher levels of JA under drought and salinity stress conditions

Jasmonic acid and its metabolites are collectively known as jasmonates and play important roles in plant development and stress responses (Wasternack *et al* ., 2013). FAD3 mediates unsaturation of linoleic acid to produce α-linolenic acid. Since α-linolenic acid is a precursor for JA biosynthesis, JA levels were quantified in mock-inoculated, vector-infected, S-FAD3 as well as OE-FAD3 plants under well-watered and also under drought and salinity stress conditions. JA levels were not significantly altered in mock-inoculated, vector-infected and S-FAD3 plants under well-watered, drought and salinity stress conditions. Compared with vector-infected plants, GmFAD3 overexpressed plants showed approximately 3-fold and 6-fold higher levels of JA under well-watered, and drought and salinity stress conditions, respectively (Figure 5a). These results indicate that JA plays important roles in drought and salinity stress tolerance in soybean

3.9 Increase in proline level in FAD3 overexpressing plants under drought and salinity stress conditions

Earlier reports demonstrated that plants accumulate proline in response to drought and salinity stress (Trovato *et al* . 2008, Goyal*et al.* 2010). Hence, we measured proline level in mock-inoculated, vector-infected, S-FAD3 and OE-FAD3 plants under well-watered, drought and salinity stress conditions. Interestingly, the level of proline accumulated in OE-FAD3 plants under both drought and a salinity stress condition was much higher than that in mock-inoculated, vector-infected and S-FAD3 soybean plants (Figure 5b). The result showed that the level of proline accumulation increased markedly in OE-FAD3 plants compared to mock-inoculated, vector infected soybean plants under drought and salinity stress condition. Under well-watered condition, the proline level was 2.17 ± 0.35 mg/g FW, 1.94 ± 0.43 mg/g FW, 2.61± 0.54 mg/g FW, 2.77 ± 0.53 mg/g FW, respectively in mock-inoculated, vector-infected, S-FAD3 and OE-FAD3 plants. Under drought stress conditions, proline content increased from 2.17 ± 0.35 mg/g FW to 3.04 ± 0.45 mg/g FW, 1.94 ± 0.43 mg/g FW to 3.43 ± 0.48 mg/g FW, 2.61 ± 0.54 to 4.01 ± 0.54 mg/g FW, 2.77 ± 0.53 to 6.79 ± 0.48 mg/g FW, respectively in mock-inoculated, vector-infected, S-FAD3 and OE-FAD3 plants. When mock-inoculated, vector-infected, S-FAD3 and OE-FAD3 plants were subjected to salt stress at 150 mM of NaCl for 4 days, proline content increased from 2.17 ± 0.35 mg/g FW to 4.1 ± 0.48 mg/g FW, 1.94 ± 0.43 mg/g FW to 4.3 ± 0.55 mg/g FW, 2.61 ± 0.54 to 5.5 ± 0.65 mg/g FW, 2.77 ± 0.53 to 7.5 ± 0.55 mg/g FW, respectively.

3.10 Increased expression of *GmWRKY* transcription factor in FAD3 overexpressed plants under drought and salinity stress

Differential tolerance to abiotic stress was achieved in Arabidopsis when transformed with *GmWRKY* transcription factor (Zhou *et al* ., 2008). This prompted us to study the expression of *WRKY*transcription factor in OE-FAD3 plants. Mock-inoculated, vector-infected, S-FAD3 and OE-FAD3 plants were analyzed for expression of *WRKY54* transcription factor under well-watered, drought and salinity stress conditions. Semi-quantitative RT-PCR analysis revealed higher expression of *GmWRKY54* transcription factor in OE-FAD3 plants as compared to mock-inoculated, vector-infected and S-FAD3 soybean plants under drought and salinity stress condition. Interestingly, in the plants which were without any stress and under well-watered conditions, expression of *GmWRKY-54* transcription factor was almost similar in mock-inoculated, vector-infected, S-FAD3 and OE-FAD3 plants (Figure 5c).

4 DISCUSSION

Abiotic stresses such as drought, salinity and low/high temperature severely affect growth, development and yield potential of crops. Therefore, it is crucial to understand abiotic stress responses in soybean in order to enhance stress resilience to maintain genetic yield potential. Fatty acids are vital constituents of cellular membrane architecture. The cell membrane acts as a prime sensor for environmental stresses and

its stabilization is essential for the survival of the plant (Zhang *et al* ., 2005; Shi *et al* ., 2008). Membrane stabilization and maintenance of its integrity are largely affected by lipid composition and the degree of fatty acid desaturation (Mikami & Murata., 2003; Shi *et al* ., 2008). Therefore, fatty acid desaturation by fatty acid desaturases by an increase in linolenic acid (C18:3) is considered one of the factors involved in the tolerance of plants to multiple abiotic stresses (Upchurch., 2008; Lenka *et al* ., 2011). Fatty acid desaturase 3 is known to mediate conversion of linoleic to ɑ-linolenic acid, a polyunsaturated fatty acid whose levels are altered under abiotic stress conditions (Napier *et al* ., 1999). In the present study, the transcript levels of the *GmFAD3*gene were manipulated in soybean by overexpression as well as silencing using a BPMV-based viral vector. Overexpression of the *GmFAD3*gene was verified by semi-quantitative RT-PCR, RT-qPCR as well as by Northern blot analysis. The size difference in *GmFAD3* in Northern blot can be explained as a fusion of BPMV-RNA2 sequence to the*FAD3* transcript. In the present study, overexpression of FAD3 employing BPMV-based viral vector resulted very high level of ɑ-linolenic acid which in turn resulted drought and salt stress tolerance although tolerance to heat was reduced. Im *et al.*(2002) reported that tobacco plants with antisense expression of omega-3 fatty acid desaturase from Arabidopsis had reduced salt tolerance. Shi*et al* . (2018) reported that overexpression of *Chorispora bungeana* microsomal *ω-3 ΦAΔ3* gene (*CbFAD3*) increased linolenic acid (C18:3) in both leaves and roots which in turn enhanced plant tolerance to drought and salt stresses in tobacco and correlated it with activation of reactive oxygen species scavenging system, plasma membrane Ca^{2+}-ATPase and stress-induced Ca^{2+} signaling. Similarly, yeast transformed with ω-6 desaturases from sunflower had increased salt tolerance (Upchurch., 2008).

Overexpression of *GmFAD3* enhanced drought and salt stress tolerance in soybean although heat stress tolerance was reduced, while FAD3 silenced plants were tolerant to heat stress but found vulnerable to drought as well as salinity stress. These results may be explained based on changes occurred at physiological, biochemical and molecular levels under non-stress and stress conditions. In the present investigation, it was observed that OE-FAD3 soybean plants showed significantly higher chlorophyll content, chlorophyll fluorescence (Fv/Fm), RWC as compared to mock-inoculated, vector-infected and S-FAD3 soybean plants under drought and salinity stress conditions. Less decrease in chlorophyll content and leaf SPAD value were observed in OE-FAD3 plants as compared to mock, vector control, S-FAD3 plants under drought and salinity stress conditions. The ability of OE-FAD3 soybean plants to tolerate drought and salinity stress could be associated with chlorophyll content and protection of chlorophyll from degradation under drought and salt stress conditions. Leaf chlorophyll content is considered to be a good indicator of photosynthetic capability in terms of Photosystem-II (PS-II, Fv/Fm) efficiency. Kumar *et al.* (2017) reported that photosynthetic efficiency in soybean was closely associated with canopy greenness reflected by higher chlorophyll content. Decrease in photosynthetic efficiency (PS-II) due to lower chlorophyll content may be the reason for reduced drought and salt stress tolerance in mock-inoculated, vector-infected, S-FAD3 plants than that of OE-FAD3 soybean plants under drought and salinity stress conditions. Jamil *et al.* (2007) also reported reduction in chlorophyll content in radish due to salt stress. Water status of plants is also crucial for plants response to various abiotic stresses especially drought and salinity. FAD3 overexpressing plants showed relatively higher RWC compared to mock-inoculated, vector-infected and S-FAD3 plants under well-watered, drought and salinity stress condition. It was observed that RWC decreased markedly in mock-inoculated, vector-infected and S-FAD3 plants as compared to OE-FAD3 plants. The decrease in RWC could be due to low water availability under drought and salt stress conditions. Drought and salinity stress induced a reduction in the relative water content indicated a loss of turgor that resulted in limited water availability for cellular process in mock-inoculated, vector-infected and S-FAD3 plants as compared to OE-FAD3 soybean plants.

Canopy temperature was found lower in OE-FAD3 plants as compared to mock-inoculated, vector-infected and S-FAD3 soybean plants under well-watered, drought and salt stress conditions. Several researchers demonstrated canopy temperature as an indicator to assess variation in transpiration rate and stomatal conductance in crop plants (Jones*et al* ., 2002; Rebetzke *et al* ., 2013; Kumar *et al* ., 2017). Lower canopy temperature could be one of the reasons for higher stomatal conductance, transpiration rate and assimilation rate in OE-FAD3 plants compared to mock-inoculated, vector-infected and S-FAD3 plants under drought and salinity stress conditions. Under well-watered conditions, S-FAD3 soybean plants showed higher chlorophyll

content, stomatal conductance, transpiration rate and carbon assimilation rate compared to mock-inoculated, vector-infected and OE-FAD3 soybean plants. Higher transpiration, stomatal conductance and carbon assimilation rate could be the reason for higher yield in S-FAD3 soybean plants compared to mock-inoculated and vector-infected as we reported earlier (Singh*et al.,* 2011) under well-watered conditions. FAD3-silenced and FAD3 overexpressed plants showed almost similar yield potential under well-watered conditions. It was also observed that yield penalty under drought and salinity stress conditions was almost similar in FAD3 silenced and FAD3 overexpressing plants.

The FAD3A overexpression resulted in higher JA levels in soybean under drought and salinity stress conditions. This result suggested important roles of jasmonate in coping with drought and salt stress. Regulation of JA synthesis is altered in stressed as well as non-stressed plants, which is associated with a variety of metabolic pathways including signal transduction and abiotic stress responses (Ahmad *et al.* , 2016). A large-scale expression profiling in barley revealed considerable overlapping for genes regulated by salinity stress and JA application (Walia *et al* ., 2007). Several researchers reported enhanced drought stress tolerance upon exogenous application of MeJA, for instance, in tobacco by improving Fv/Fm, alleviating degradation of chlorophyll and protecting PSII under drought stress (Wei-Wei *et al.,* 2011), in wheat by increasing photosynthesis rate, delayed senescence and improving water status (Ma *et al.* 2014). Similarly, salinity stress tolerance was also improved by exogenous application of MeJa in several plants, for example, in tomato (Enteshari & Jafari., 2013) and in soybean by improving photosynthesis, transpiration rate, chlorophyll and proline content (Yoon *et al* ., 2009). *Arabidopsis thaliana* plants with duplication of a genomic region having *FAD3* locus had elevated levels of linolenic acid, a precursor of JA, content in seed oil (O'Neill *et al* ., 2011). It may be worthy of mentioning here that JA biosynthesis involves two pathways; via an octadecanoid pathway involving addition of molecular oxygen to linolenic acid or a hexadecanoid pathway beginning from the precursor oleic acid.

Proline is one of the most important organic compatible solutes which protect plants against free radical-induced damage under stress condition. A large number of plant species accumulate proline in response to stresses, such as salinity and drought (Trovato *et al* ., 2008). In the present study, it was observed that drought and salinity stresses resulted in significant increase in proline content. In line with present investigation, rice roots exposed to NaCl stress resulted in accumulation of proline with increasing NaCl concentrations (Morant *et al* ., 2004). Increasing proline content under drought and salinity stress might be due to activation of proline syntheses from glutamate or decrease in its utilization in protein syntheses. Gad (2005) reported that proline may be the major source of energy and nitrogen during immediate post stress metabolism and accumulated proline supplies energy for growth and survival, thereby inducing drought and salinity stress tolerance. Taken together, enhanced drought and salinity stress tolerance in OE-GmFAD3 plants may be correlated with C18:3 induced membrane stabilization, higher JA level and the increased expression of stress-responsive genes such as *WRKY54* .

In the present study, OE-FAD3 plants showed higher expression of*WRKY54* transcription factor as compared to mock-inoculated, vector-infected and S-FAD3 soybean plants under drought and salinity stress conditions. The JA-responsive TFs like *WRKY* regulate the expression of many genes involved in the growth and development of plants, and especially the responses and adaptation of plants to the environment (Supplementary Fig. S2). The WRKY transcription factors play pivotal role in the regulation of abiotic stress responses in plants. The *WRKY* gene involved in multiple pathways induced by stresses and JA signal network (Finatto *et al.,* 2018). Zhou *et al* . (2008) reported deferential abiotic stress tolerance by transforming Arabidopsis with *GmWRKY* transcription factors. In*Arabidopsis thaliana* , overexpression of *GsJAZ* , a novel JAZ family gene from *Glycine soja* , enhanced the salt and alkali stress tolerance (Zhu *et al* ., 2012). It may be worth noting that JAZ proteins act as repressors of JA signaling. The endogenous bioactive form of JA, a JA-isoleucine conjugate (JA-Ile) mediates the binding of Jasmonate ZIM (JAZ) proteins to the F-box protein CORONATINE INSENSITIVE1 (COI1) and forms the Skp1/Cullin/F-box (SCFCOI1) complexes (Fig. S2). Upon degradation of JAZ proteins via the 26S proteasome pathway, transcription factors, including WRKY, MYC, bHLH/MYB, are relieved from JAZ-proteins and activate their respective downstream responses (Cheng *et al* ., 2011; Song *et al* ., 2011; Qi*et al.,* 2011 (Supplementary Fig. S2). In rice, a

signaling module consisting of *OsbHLH148 –OsJAZ–OsCOI1* mediates jasmonate-regulated gene expression under drought stress. Jasmonate mediated degradation of *OsJAZs* and activation of *OsbHLH148*leads to downstream drought stress responses (Seo *et al* ., 2011). Protection of OE-FAD3 soybean plants against drought and salinity stresses was observed in the investigation of physiological, biochemical and molecular changes. The enhanced tolerance of the OE-FAD3 plants to drought and salinity stress may be due to higher chlorophyll content, protection of PS-II, higher water retention capacity, lower canopy temperature, higher transpiration, by increased level of JA under drought and salinity stress condition as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants.

GmFAD3A overexpression plants were vulnerable to heat stress while plants silenced for FAD3A exhibited heat tolerance. In line with present investigation, tobacco plants silenced for chloroplast ω-3 desaturase had very low levels of trienoic fatty acids and exhibited resistance to elevated temperature (36^{o}C) when compared to control plants (Upchurch, 2008). In soybean, several studies have reported reduced linoleate and linolenate levels and elevated oleate levels when exposed to higher temperatures during seed development (Upchurch., 2008). It may be worth noting that, during a genome wide expression profiling in wheat cultivars with differential thermal tolerance, down-regulation of FAD3 was observed when subjected to heat stress of 40^{o}C for 1 hour, which was preceded by heat acclimation of 34^{o}C for 3 hours (Qin *et al* ., 2008). In agreement with present study, *Arabidopsis thalianafad7fad8* double mutants exhibited thermal tolerance (Zhang*et al* ., 2005). Probably a FAD3 mediated modulation of membrane fluidity plays a role in thermal tolerance.

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Conflict of Interest

Authors declare no competing interests

Author contribution

AKS, RKV and AK planned and designed the research; AKS, MK, SKR performed experiment; JR, MBR provided resources; AKS, JR, RKV and AK analyzed data and wrote the manuscript.

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Table 1: List of primers used for RT-qPCR analysis of mock-inoculated, vector- infected, S-FAD3, and OE-FAD3 soybean plants

S.No.	Primer name	Primer sequence
1	FAD3-OE-Real-Time PCR-For	5'-AACTGGCTTCTCTGGCTAATC-3'
2	FAD3-OE-Real-Time PCR-Rev	5'-GTGTCCCACCAGGCTATTT-3'
3	FAD3-Sil-Real-Time PCR-For	5'-CACTGTGCCATTTCCATTGTT-3'
4	FAD3-Sil-Real-Time PCR-Rev	5'- GGTGGGAACAGATTGCTGTA-3'
5	*G max*-β-Tubulin- Real-Time PCR -For	5'-TTGATCTCCGCAACCATGAG-3'
6	*G max*-β-Tubulin- Real-Time PCR -Rev	5'-GAGGGAAAGGGATGAGATTCAC-3'

Figure legends

Figure 1 Expression analysis of the GmFAD3 in the leaf tissue. (a) Reverse-transcription polymerase chain reaction analysis showing expression of the *GmFAD3* in the leaf tissue from mock-inoculated (M), vector-infected (V) and *GmFAD3* -overexpressed (OE-FAD3) soybean plants. (b) Real Time Polymerase Chain Reaction analysis showing relative mRNA level of the *GmFAD3* in the leaf tissue from mock (M), vector-infected (V), GmFAD3-silenced (S-FAD3) and*GmFAD3* -overexpressed (OE-FAD3) soybean plants. Values were represented as mean ± standard errors. Each experiment was repeated three times. Significant differences among the mean values were compared using by Student's t Test ($P < 0.001$). Asterisk denotes significant difference. (c) Morphological phenotype of leaves from of vector infected, *GmFAD3* silenced (S-FAD3) and FAD3-overexpressing (OE-FAD3) soybean plants. (d) RNA blot analysis of mock-inoculated (M), vector-infected (V) and GmFAD3 overexpressing plants (OE-FAD3). Ethidium bromide staining of rRNA was used as a loading control. RNA blot was probed with GmFAD3A probe.

Figure 2 Response of FAD3 silenced and overexpressing plants to drought, salinity and thermal stress and expression analysis of *GmWRKY54*transcription factor. (a) Difference in drought tolerance at the whole plant level between mock-inoculated (M), vector-infected (V), GmFAD3-silenced (S-FAD3) and *GmFAD3* -overexpressed (OE-FAD3) soybean plants. Watering was withheld for 3 days. (b) Difference in salinity stress tolerance (150 mM NaCl) at the whole plant level between mock, vector alone, FAD3-silenced and FAD3 overexpressing soybean plants. (c) Difference in thermal-tolerance at the whole plant level between mock, vector alone, FAD3-silenced and FAD3 overexpressing soybean plants. Values were represented as mean ± standard errors. Each experiment was repeated three times. Significant differences among the mean values were compared using by Student's t Test ($P < 0.001$). Asterisks denote significant difference.

Figure 3 Effect of drought and salinity stress on various physiological processes in mock (M), vector-infected (V), GmFAD3-silenced (S-FAD3) and GmFAD3-overexpressed (OE-FAD3) soybean plants. Effect of drought and salinity stress on chlorophyll content (a), SPAD value (b), Chlorophyll fluorescence (Fv/Fm) value (c), relative water content (d) in mock (M), vector-infected (V), GmFAD3-silenced (S-FAD3) and GmFAD3-overexpressed (OE-FAD3) soybean plants. Values were represented as mean ± standard errors. Each experiment was repeated three times. Significant differences among the mean values were compared using by

Student's t Test ($P < 0.05$).

Figure 4 Effect of drought stress and salinity stress on canopy temperature (a), transpiration rate (b), stomatal conductance (c), and carbon assimilation rate (d) in mock (M), vector-infected (V), GmFAD3-silenced (S-FAD3) and GmFAD3-overexpressed (OE-FAD3) soybean plants. Values were represented as mean ± standard errors. All the experiments were repeated at least three times. Significant differences among the mean values were compared using by Student's t Test ($P < 0.05$).

Figure 5 Effect of drought stress and salinity stress on JA level in mock (M), vector-infected (V), GmFAD3-silenced (S-FAD3) and GmFAD3-overexpressed (OE-FAD3) soybean plants under non stress (well-watered), drought and salinity stress conditions (150 mM) NaCl (a), (b) Proline content in mock (M), vector-infected (V), GmFAD3-silenced (S-FAD3) and GmFAD3-overexpressed (OE-FAD3) soybean plants under drought, salinity, well-watered conditons (b). Values were represented as mean ± standard errors. All the experiments were repeated at least three times. Significant differences among the mean values were compared using by Student's t Test ($P < 0.05$). (c) Reverse-transcription polymerase chain reaction analysis showing expression level of *WRKY54* transcription factor in mock, vector control, FAD3 silenced and FAD3 overexpressing soybean plants under well-watered, drought and salinity stress conditions. Expression of β-tubulin was used to check equal quantity loading of cDNA.

Supplementary Figures

Figure S1. Scheme of the jasmonic acid biosynthesis pathway. The enzymes and the intermediates are indicated. Jasmonic acid regulates various physiological processes and triggers defense response as well as responses to abiotic stresses.

Figure S2. The regulatory network of the jasmonic acid signaling pathway. Abiotic stresses induce the synthesis of JA, which can be converted to the biologically active JA-Ile by JAR1. Perception of JA-Ile by its receptor COI1 triggers the degradation of JAZ proteins, leading to the release of downstream transcription factors and the regulation of JAs-responsive genes such as WRKY54 in various processes. SCF: Skp1, Cullin and F-box proteins; COI1: Coronatine Insensitive 1; JAZ: jasmonate ZIM-domain protein; TF: transcription factor; 26S: 26S Proteasome.

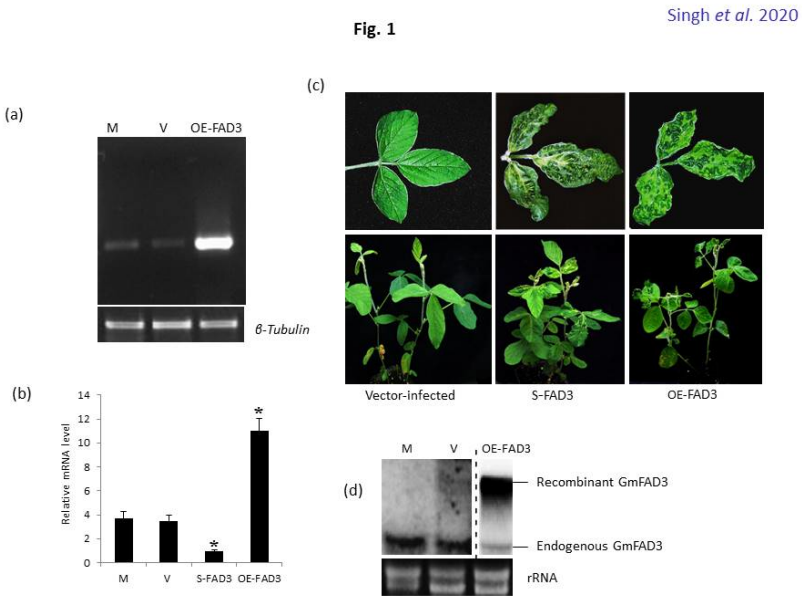

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Fig. 2

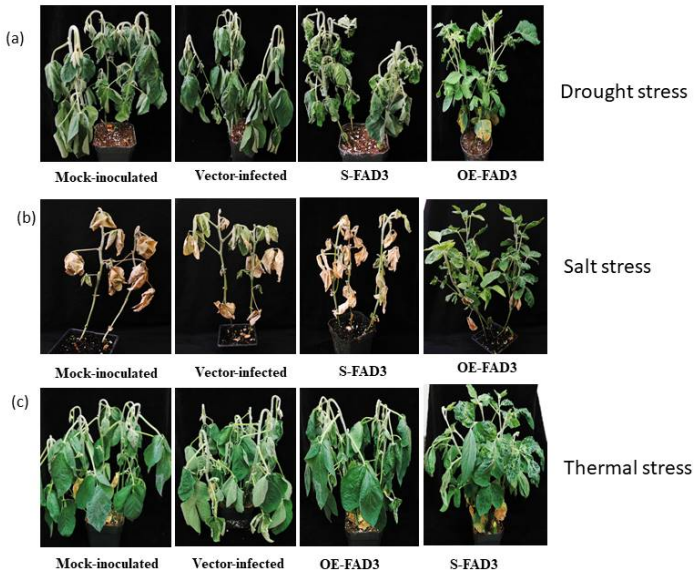

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Fig. 3

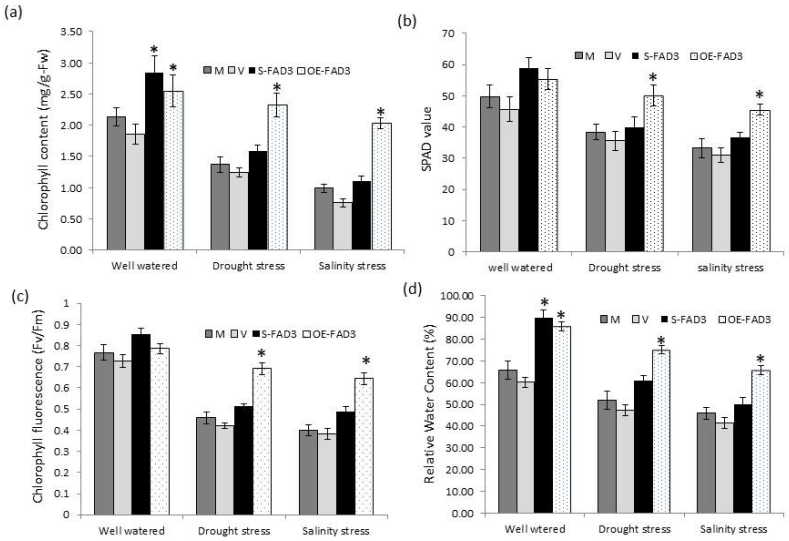

Fig. 4

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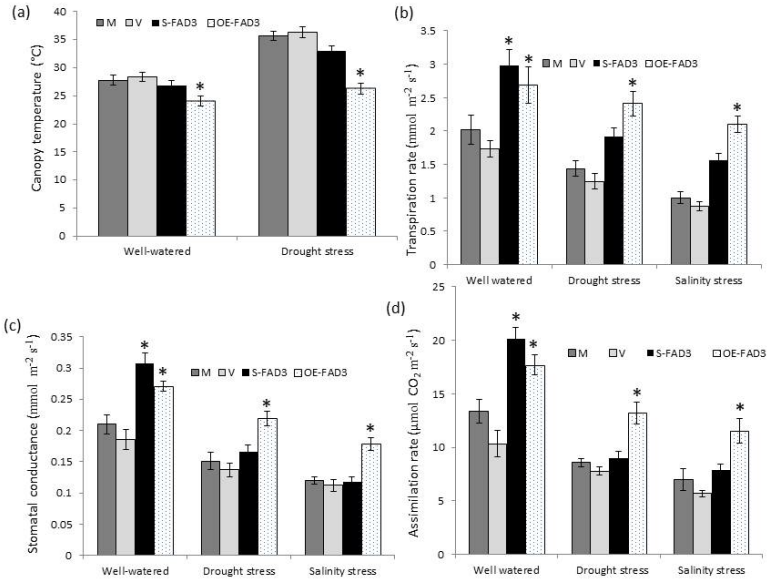

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Fig. 5

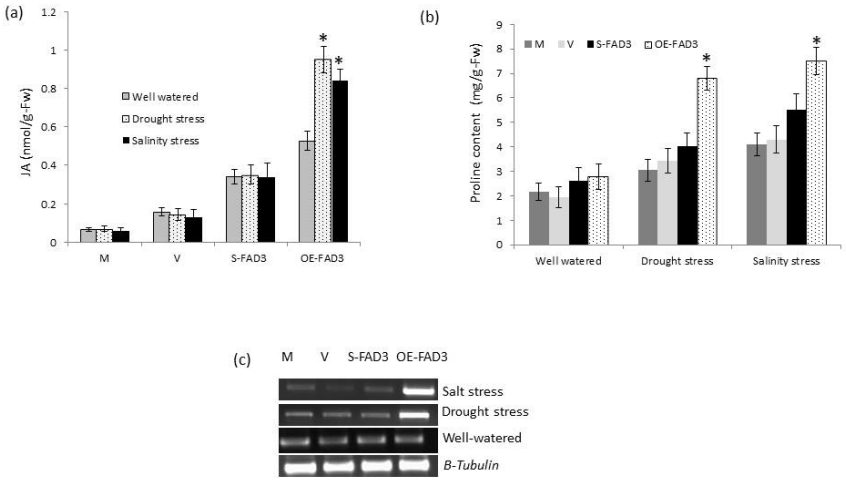

Fig. S1

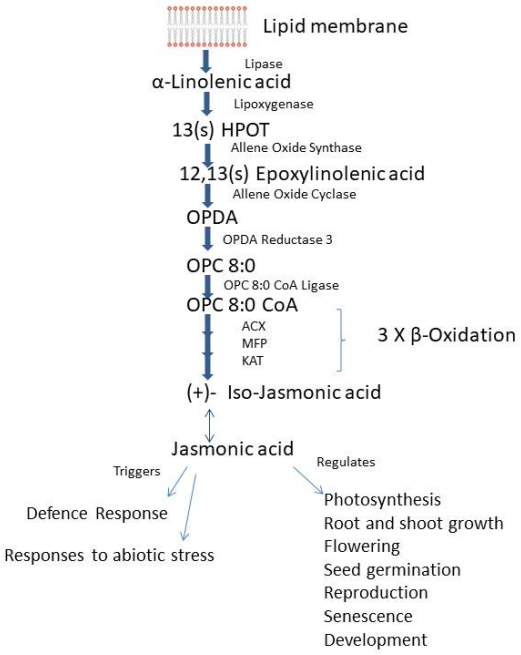

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Fig. S2