Modulation of GmFAD3 expression alters abiotic stress responses in soybean

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Abstract

**Key message** This study focused on enhancing resilience of soybean crops to drought and salinity stresses by overexpression of GmFAD3A gene, which plays an important role in modulating membrane fluidity and ultimately influence plants response to various abiotic stresses.

**Abstract** Fatty acid desaturases (FADs) are a class of enzymes that mediate desaturation of fatty acids by introducing double bonds. They play an important role in modulating membrane fluidity in response to various abiotic stresses. However, a comprehensive analysis of GmFAD3 in drought and salinity stress tolerance in soybean is lacking. We used bean pod mottle virus (BPMV)-based vector for achieving rapid and efficient overexpression as well as silencing of Omega-3 Fatty Acid Desaturase gene from Glycine max (GmFAD3) to assess the functional role of GmFAD3 in abiotic stress responses in soybean. Higher levels of recombinant BPMV-GmFAD3A transcripts were detected in overexpressing soybean plants. Overexpression of GmFAD3A 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. The GmFAD3A-overexpressing plants showed higher levels of chlorophyll content, efficient photosystem-II, relative water content, transpiration rate, stomatal conductance, 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 the current study revealed that GmFAD3A-overexpressing soybean plants exhibited tolerance to drought and salinity stresses. However, soybean plants silenced for GmFAD3 were vulnerable to drought and salinity stresses.

**Keywords** Fatty acid desaturase · Overexpression · Gene silencing · Jasmonic acid · WRKY transcription factor · Drought stress · Salinity stress · Bean pod mottle virus

Introduction

Soybean [Glycine max (L.) Merr] is an important oilseed crop contributing to the protein and oil requirement of humans as well as animals. It is also used as a raw product for human health and industrial applications. Therefore, the demand of soybean is increasing continuously worldwide. Growth, development, reproduction and survival of plants are greatly compromised by abiotic stresses such as drought and salinity which may occur individually or in combination (Choudhury et al. 2017; Gupta et al. 2020; Lamers et al. 2020; Rane et al. 2021a, b, c). Drought and salinity also cause severe losses to soybean productivity worldwide by adversely affecting plant growth, development and yield potential (He et al. 2017; Mutava et al. 2015)). Thus, enhancing resilience of soybean crops to abiotic stress in order to maintain genetic yield potential is extremely demanding areas in agricultural research.

Drought stress could affect physiological processes such as lowering photosynthesis, transpiration rate and stomatal conductance (Jaleel et al. 2009). Root always plays...
an important role in regulating crop productivity under drought stress (Bengough et al. 2011; Prince et al. 2017). Plants with deeper roots assimilate more water and nutrition under drought stress (Hammer et al. 2009). Salinity is another vital limiting factor for sustainable agriculture with depressing crop growth and production (Munns and Gillham 2015; Negrao et al. 2017). Salinity stress could not only reduce crop yield by affecting several physiological processes (Stavridou et al. 2017), but also could reduce the ability of roots to take up water and nutrition (Munns 2002; Han et al. 2014).

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 an important role during signal transduction (Wang 2004). Linoleic acid and α-linolenic acid are crucial polyunsaturated fatty acids for most plant seed oils (Napier and Graham 2010). The role of linolenic acid as a stress signaling compound 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 (Wasternack 2007; Wang et al. 2021).

Commodity soybean oil contains about 10% palmitic acid, 4% stearic acid, 18% oleic acid, 55% linoleic acid and 13% linolenic acid. Genes controlling contents of oleic acid and polyunsaturated fatty acids have been reported in soybean (Pham et al. 2012). Fatty acid desaturase 3 (FAD3) enzymes convert linoleic acid to α-linolenic acid and this enzymatic process involves three active members viz. FAD3A, FAD3B and FAD3C (Bilyeu et al. 2003; Singh et al. 2011). However, GmFAD3A has been reported to have a higher expression during embryogenesis and likely being the major contributor of 18:3 content in soybean oil (Bilyeu et al. 2003; Singh et al. 2011). The GmFAD3A nucleotide sequences were analyzed for identity and compared with GmFAD3B and GmFAD3C sequences (Supplementary Fig. 1).

In tobacco plants, expression of FAD3/FAD8 enhanced tolerance to osmotic stress (Zhang et al. 2005). In another study, silencing FAD7 in tobacco plants reduced the levels of linolenic acid and tolerance to drought and salinity stress (Im et al. 2002). Shi et al. (2018) isolated microsomal α-3 FAD gene from Chorispora bungeana (CbFAD3). The expression pattern and the functionality of CbFAD3 were analyzed in C. bungeana suspension-culture cells and yeast cells. Shi et al. (2018) also elucidated function of CbFAD3 in transgenic tobacco plants expressing CbFAD3 gene. The overexpression of CbFAD3 gene conferred tolerance to drought and salinity stresses in tobacco plants through an integrated regulation that involves increase in membrane stability, photosynthetic capacity and the expression of stress-responsive genes (Shi et al. 2018). In recent years, some novel genes/transcription factors modulating various abiotic stress responsive genes have been characterized 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 modulating expression of several genes/transcription factors (Jumra and Bhatia 2019; Zhang et al. 2019; Chen et al. 2019). Expression patterns of WRKY transcription factors from soybean in response to different abiotic stresses were characterized and found to have differential effect on abiotic stress tolerance in transgenic Arabidopsis plants (Zhou et al. 2008). Hence, in the present investigation, the role of WRKY transcription factors in responses to various abiotic stresses in soybean was elucidated. Previously, Singh et al. (2011) silenced FAD3 employing BPMV-based VIGS vector and FAD3-silenced soybean plants were analyzed for biotic stress responses, but plants response to abiotic stress was not studied. Flores et al. (2008) silenced FAD3 gene employing siRNA-mediated approach in soybean, however, a comprehensive analysis for their role in drought and salinity stress responses was not carried out. In the present investigation, we assessed the functional role of GmFAD3A to abiotic stress responses in soybean and it was observed that the GmFAD3A-overexpressing plants exhibited tolerance to drought and salinity stresses. On the contrary, GmFAD3-silenced soybean plants were vulnerable to drought and salinity stresses.

Materials and methods

Construction of BPMV-based viral vectors for overexpression of GmFAD3A and silencing FAD3 gene in soybean

For construction of BPMV-based viral vector for overexpression of GmFAD3A gene in soybean, a full-length coding sequence (1128 bp) without stop codon of GmFAD3A gene was PCR amplified using soybean cDNA as template with primers (forward primer- 5′-AAAAACGCCTATGGTTAAA GACACAAAG-3′ and reverse primer- 5′-AAAGGCCT GTGTCGTGCGAGTGGAG-3′). Primers were designed to amplify full-length coding sequence of GmFAD3A as retrieved from the database (AY204710). In the present investigation, BPMV-based viral vector (Zhang and Ghabrial 2006) comprising of pGHopRNA1-BPMV harbouring native BPMV-RNA1 (Fig. 1a) and pGG7RNA2-BPMV (Fig. 1b) was used for cloning GmFAD3A gene. For construction of GmFAD3A overexpression vector, the PCR product representing full-length coding sequence without stop codon of GmFAD3A digested with StuI was cloned into MscI-digested pGG7RNA2-BPMV vector. The BPMV-based viral vector for overexpressing GmFAD3A
was designated as pGG7RNA2-BPMV-OE-GmFAD3A (Fig. 1c). Construction of FAD3 silencing vector was described previously (Singh et al. 2011). In vitro transcripts from native BPMV-RNA1 and recombinant BPMV-RNA2 are required together for overexpressing GmFAD3A gene employing BPMV-based viral vector in soybean. a The pGHopRNA1-BPMV vector used for in vitro transcript preparation. The BPMV RNA1 encodes Helicase and RNA dependent RNA Polymerase (RdRp). The BPMV-RNA1 was cloned under control of the T7 promoter (T7Pr). b The pGG7RNA2-BPMV vector used for in vitro transcript preparation. B. c The pGG7RNA2-GmFAD3A-BPMV vector used for in vitro transcript preparation. The full-length cDNA of GmFAD3A without stop codon (1128 bp) was inserted into pGG7RNA2-BPMV vector using MscI restriction site.

**Fig. 1** Schematic presentation of BPMV-based viral vector used for overexpression of GmFAD3A gene in soybean. In vitro transcripts from native BPMV-RNA1 and recombinant BPMV-RNA2 are required together for overexpressing GmFAD3A gene employing BPMV-based viral vector in soybean. a The pGHopRNA1-BPMV vector used for in vitro transcript preparation. The BPMV RNA1 encodes Helicase and RNA dependent RNA Polymerase (RdRp). The BPMV-RNA1 was cloned under control of the T7 promoter (T7Pr). b The pGG7RNA2-BPMV vector used for in vitro transcript preparation. B. c The pGG7RNA2-GmFAD3A-BPMV vector used for in vitro transcript preparation. The full-length cDNA of GmFAD3A without stop codon (1128 bp) was inserted into pGG7RNA2-BPMV vector using MscI restriction site.

**Plant materials, growing conditions, in vitro transcription and plant inoculation with in vitro transcripts**

Soybean cultivars, viz., Essex, Harosoy, Williams, NRC-37 and JS-335 were grown in the greenhouse with day and night temperatures of 27 and 24 °C, respectively. In vitro transcripts were prepared as described previously (Zhang and Ghabrial 2006; Diaz-Camino et al. 2011; Singh et al. 2011; Kachroo and Ghabrial 2012; Rao et al. 2014; Shine et al. 2016). Soybean plants at unrolled unifoliolate leaves (VC-stage) were dusted with carborundum followed by rub-inoculation with in vitro transcripts derived from plasmid pGHopRNA1-BPMV containing a full-length infectious BPMV-RNA1 cDNA and empty vector plasmid pGG7RNA2-BPMV, recombinant pGG7RNA2-S-FAD3-BPMV viral vector harbouring FAD3 silencing fragment, and pGG7RNA2-OE-GmFAD3A-BPMV having a full length coding sequence of GmFAD3A gene without stop codon, respectively for generating vector-infected control plants, FAD3-silenced and GmFAD3A-overexpressing plants. In vitro transcripts were prepared separately from plasmid pGHopRNA1-BPMV, pGG7RNA2-BPMV, pGG7RNA2-BPMV-S-FAD3 and pGG7RNA2-OE-GmFAD3A. In vitro transcripts prepared from pGHopRNA1-BPMV was mixed separately with transcripts derived from pGG7RNA2-BPMV, pGG7RNA2-S-FAD3-BPMV and pGG7RNA2-OE-FAD3-BPMV before rub-inoculation on VC-stage soybean plants for generating vector-infected,
FAD3-silenced and GmFAD3A-overexpressing plants, respectively. Soybean plants raised through in vitro transcripts inoculation were used to verify silencing FAD3A,B,C genes or overexpression of GmFAD3A gene. Soybean plants with four unfolded trifoliate leaves which were previously inoculated on the unifoliolate leaves with fine ground freeze-dried leaves of vector-infected, FAD3-silenced and GmFAD3A-overexpressing plants raised from in vitro transcripts inoculation, were used to study response of soybean plants to drought and salinity stresses at morphological, physiological, biochemical and molecular levels. The experiments were replicated three times and plants used for testing abiotic stress responses were verified for GmFAD3A mRNA level by RT-qPCR.

Estimation of growth parameters and biomass

Mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing soybean plants at V3-growth stage were gently uprooted from pots and washed carefully with tap water and then plant height and root length were measured. For measurement of biomass (dry weight), shoots and roots were subsequently separated and oven dried at 65 °C for 72 h.

RNA extraction, quantitative Real-Time PCR and RNA blot analyses

Total RNA extraction from leaf tissues of mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing plants 21 days post-infection was performed using RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) as per manufacturer’s instructions. First-strand cDNA synthesis was performed using SuperScript® II Reverse Transcriptase (Invitrogen). Relative differences in GmFAD3A transcript accumulation in mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing soybean plants were evaluated by quantitative RT-PCR (RT-qPCR) using forward primer 5′-CAGTCGGCCATTCGCCATGTCG-3′ and reverse primer 5′-GTTGAGACACAGATTGCTGTA-3′. Transcript accumulation of GmWRKY13, GmWRKY19, GmWRKY21, GmWRKY39, GmWRKY54 and GmWRKY62 transcription factors in mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing plants under well-watered (no stress), drought and salinity stress conditions were evaluated by RT-qPCR using set of primers as mentioned in Supplementary Table 1. The 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. The relative mRNA levels of GmFAD3A and GmWRKY19 transcription factors were determined by normalizing the PCR threshold cycle number with that of β-Tubulin. All experiments were repeated three times independently and the average was calculated.

RNA blot hybridization was achieved by electrophoresis of total RNA 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 hybridized with a [32P]-dCTP-labeled probe (GmFAD3A gene 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 band intensity was quantified using the Image-Quant software (Molecular Dynamics).

Assessment of tolerance to drought and salinity stresses in FAD3-silenced and GmFAD3A-overexpressing soybean plants

Nine individual plants of soybean cultivars, i.e., Essex, Harosoy, Williams, NRC-37 and JS-335 were tested for checking efficacy of silencing FAD3 as well as overexpression of GmFAD3A gene. Tolerance to drought stress at whole plant level in mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing soybean plants, cv. Essex was evaluated by uniformly withholding watering for 4 days under greenhouse conditions. For salinity stress tolerance assessment, mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing soybean plants, cv. Essex were watered with 100 and 150 mM NaCl for four days with three days intervals.

Moisture measurements in pot experiment

Mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing soybean plants were exposed to drought stress by withholding watering for four days in greenhouse. The soilrite samples from pots for drought stress and well-watered (no stress) treatments were collected in aluminum boxes with lids fitted securely. The samples were weighed immediately after collection and oven dried at 105 °C for 72 h to determine the moisture content of soilrite by gravimetric method. Moisture content of soilrite mix was calculated using formula: Moisture content (%) = Weight of moist soilrite—weight of dry soilrite/weight of dry soilrite × 100.

Chlorophyll assay, leaf SPAD value, photosystem-II efficiency and relative water content

For chlorophyll content estimation, a 250 mg of finely-ground fresh leaf tissues of mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing...
plants was extracted with 10 ml of DMSO in a test tube and incubated at 60 °C for 6 h. Absorbance was measured at 665 nm and 649 nm using UV–VIS spectrophotometer (UV-1800, Shimadzu, Japan) and total chlorophyll content was 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 in leaves of mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing plants under no stress, drought and salinity stress conditions (100 and 150 mM NaCl). A total nine plants of soybean cultivar Essex were analyzed for SPAD value with three readings recorded from each plant. Chlorophyll fluorescence was measured in the excised leaves at V3-stage of mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing soybean plants for evaluating photosystem-II efficiency. The leaf images were captured by chlorophyll fluorescence measuring system (PSI, Czechoslovakia) and image analysis was performed using Fluorochrom7 software. The relative water content (RWC) was determined in mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing soybean plants at V3-stage under no stress and drought stress condition imposed by withholding watering for four days. The RWC was calculated using following equation, RWC = (FW–DW) × 100/(SW-DW), where FW is the fresh weight, SW is the water-saturated weight and DW is the dry weight (Turner, 1981). For RWC estimation, trifoliate leaves were excised and 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 h.

**Canopy temperature, leaf stomatal conductance and transpiration rate**

The canopy temperature was measured with a hand-held IR-Gun thermometer under no stress and drought stress condition imposed by withholding watering for four days at V3-stage of mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing soybean plants. The data were recorded approximately a 50 cm above the canopy. A porometer (Delta-T AP4, Delta-T Devices, Cambridge, UK) was used to estimate stomatal conductance of leaves with 3rd trifoliate leaf of mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing plants and nine replicates were used for measurement. Transpiration rate was measured on trifoliate leaves of mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing plants with a portable photosynthesis system (GFS-3000, WALZ) at a photosynthetic photon flux density 900 μmol m⁻² s⁻¹, and air temperature of 25 ± 1 °C to 28 ± 2 °C.

**Proline content**

Free proline content using 500 mg fresh leaf samples of mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing plants was estimated using the ninhydrin method as described by Bates et al. (1973). Optical density was measured at 520 nm using toluene as blank through UV–VIS spectrophotometer (UV-1800, Shimadzu, Japan). The amount of proline was determined from a standard curve.

**Fatty acid and jasmonic acid analyses**

Fatty acid (FA) analysis was performed as described previously (Kachroo et al. 2008). For FA analysis, leaves from 3–4 weeks-old mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing soybean plants were transferred in 2 ml of 3% H₂SO₄ in methanol containing 0.001% butylated hydroxytoluene. One ml of hexane with 0.001% butylated hydroxytoluene was added after 30 min of incubation at 80 °C. The hexane phase was then collected in vials for gas chromatography (GC), and samples were analyzed by GC on a Varian FAME 0.25 mm 650 m column and were quantified with flame ionization detection. The identities of the peaks were determined by comparing the retention times with known FA standards. Mole values were obtained by dividing peak area by molecular weight of the FA.

For extraction of jasmonic acid, one gram leaf tissues of mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing plants was ground in cold 100% methanol and dihydrojasmonic acid was used as an internal standard. The methanol extract was passed through a Sep-pak-18 column (Waters; Sep-Pak Classic C18 cartridge). The column purified extract was processed as described earlier by Xia et al. (2009) and used to inject into gas chromatograph attached to an electron ionization detector (Hewlett Packard GCD Systems).

**Statistical analysis**

Data obtained for the soil moisture, morphological, physiological, biochemical and molecular analyses were subjected to analysis of variance (ANOVA). Each experiment was repeated three times and the data shown are the average of three replicates ± Standard Errors. Significant differences among the mean values were compared using the Student’s t-Test. For fatty acid profiling of leaf, immature and mature seeds from mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing plants, experiments were repeated three times. The data depicted are the average of
three replicates ± Standard Error. One way ANOVA was used for statistical analysis of data. least significant difference (LSD) at $P < 0.05$ was calculated for making comparison of mean values.

## Results

### Higher accumulation of BPMV-RNA2-GmFAD3A recombinant transcripts in GmFAD3A-overexpressing plants

For overexpression of $GmFAD3A$, a full length cDNA (1128 bp) coding for $GmFAD3A$ lacking the termination codon was inserted in $MscI$ site of the pGG7RNA2-BPMV vector. Plants infected with recombinant vector carrying a full-length cDNA of $GmFAD3A$ showed distinct phenotype as compared to the vector-infected and FAD3-silenced soybean plants (Fig. 2a). The RT-qPCR was conducted to analyze endogenous $GmFAD3A$ or recombinant $BPMV$-RNA2:$GmFAD3A$ transcripts. The RT-qPCR analysis revealed about three-fold decrease in FAD3 mRNA levels in FAD3-silenced soybean plants, while about five-fold higher $GmFAD3A$-mRNA levels in $GmFAD3A$-overexpressing soybean plants (Fig. 2b). To further validate the transcript abundance for $GmFAD3A$, RNA blots were probed using $GmFAD3A$ specific probe (Fig. 2c). The $GmFAD3A$-overexpressing plants revealed two bands corresponding to endogenous $GmFAD3A$ transcripts and recombinant $BPMV$-RNA2:$GmFAD3A$ transcripts. In mock-inoculated and vector-infected, as expected, a single RNA band corresponding to endogenous $GmFAD3A$ was detected. Due to the strong hybridization signals from recombinant $BPMV$-RNA2:$GmFAD3A$, the exposure time for overexpression treatments was kept very short (< 30 min), while exposure time for mock-inoculated and vector infected plants was kept for 24 h. Since, exposure time was different, the autoradiogram of mock-inoculated and vector-infected control was separated from $GmFAD3A$ overexpression treatment (Fig. 2c). Because of lower exposure time, lower abundance of endogenous $GmFAD3A$ was detected in $GmFAD3A$-overexpressing plants as compared to mock-inoculated and vector-infected plants (Fig. 2c). Otherwise, level of endogenous $GmFAD3A$ was similar in mock-inoculated, vector-infected and $GmFAD3A$ overexpression treatments.

### Silencing FAD3 and overexpression of $GmFAD3A$ gene alter fatty acid profile in soybean plants

Since FAD3 protein desaturates 18:2–18:3 in soybean, we analyzed mock-inoculated, vector-infected, FAD3-silenced and $GmFAD3A$-overexpressing soybean plants for their fatty acid (FA) profiles in leaves, immature and mature seed. In line with our earlier reports (Kachroo et al. 2008; Singh et al. 2011), infection with BPMV did not significantly alter FA levels in soybean plants. The $GmFAD3A$-overexpression significantly increased 18:3 levels in leaves and immature seeds as compared to mock-inoculated and vector-infected soybean plants. On the other hand, FAD3-silenced plants exhibited significant reduction in 18:3 levels in leaf tissues as well as immature seeds as compared to mock-inoculated and vector-infected soybean plants. Leaf tissue from $GmFAD3A$-overexpressing plants showed 69.5 percent mole level of 18:3 as compared to 31.2 percent mole level in FAD3-silenced, 41.5 percent mole level in vector-infected and 42.5 percent mole level in mock-inoculated soybean plants (Table 1). Furthermore, immature seeds from $GmFAD3A$-overexpressing plants revealed 39.5 percent mole level of 18:3 compared to 19.1 percent mole level in FAD3-silenced, 28.1 percent mole level in vector-infected and 26.5 percent mole level in mock-inoculated soybean plants (Table 1). The level of 18:2 and 18:3 was almost similar in mature seed from mock-inoculated, vector-infected, FAD3-silenced and $GmFAD3A$-overexpressing soybean plants (Table 1).

### Overexpression of $GmFAD3A$ does not alter soybean seed size

Our earlier work (Singh et al. 2011) had demonstrated an increase in soybean seed size and weight in FAD3-silenced plants as compared to mock-inoculated and vector-infected plants. In the present investigation also, seed weight and seed size were higher in FAD3-silenced plants as compared to mock-inoculated, vector-infected and $GmFAD3A$-overexpressing plants (Table 2). To study the impact of $GmFAD3A$ overexpression, these seed traits were analyzed in mock-inoculated, vector-infected and $GmFAD3A$-overexpressing plants (Table 2). To study the impact of $GmFAD3A$ overexpression, these seed traits were analyzed in mock-inoculated, vector-infected and $GmFAD3A$-overexpressing plants of soybean cultivars, i.e., Essex, Harosoy, Williams, NRC-37 and JS-335. Although the $GmFAD3A$-overexpressing plants exhibited BPMV associated symptoms, they produced pods with seeds that were similar to those of mock-inoculated and vector-infected soybean plants in size and weight (Table 2).

### Moisture level under well-watered (no stress) and drought stress conditions

Moisture level was measured by gravimetric method for soilrite used to grow mock-inoculated, vector-infected, FAD3-silenced and $GmFAD3A$-overexpressing soybean plants. For imposing drought stress, watering withheld for four days in greenhouse. Under no stress condition, the moisture content of soilrite was about 30%, while under drought stress condition for four days the moisture content depleted up to 16% (Table 3). It was observed that $GmFAD3A$-overexpressing plants survived and grew normally, while mock-inoculated,
vector-infected and FAD3-silenced plants showed severe wilting phenotype at depleted moisture conditions (16%).

**Plant growth and biomass accumulation under no stress, drought and salinity stress conditions**

Plant height of mock-inoculated, vector-infected, FAD3-silenced and *GmFAD3A*-overexpressing soybean plants was not significantly different under no stress condition (Table 4). However, shoot and root biomass (dry weight) was higher in *GmFAD3A*-overexpressing soybean plants as compared to mock-inoculated, vector-infected, FAD3-silenced soybean plants under no stress, drought and salinity stress conditions (Table 4). The *GmFAD3A*-overexpressing plants had longer roots as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants under no stress, drought stress and salinity stress conditions. Salinity and drought stress resulted significant reduction in plant height, shoot and root biomass (dry weight) in mock-inoculated, vector-infected, FAD3-silenced and *GmFAD3A*-overexpressing soybean plants, cv. Essex (Table 4). However,
the GmFAD3A-overexpressing soybean plants were less impacted with respect to plant height and shoot and root biomass (dry weight) than that of mock-inoculated, vector-infected and FAD3-silenced soybean plants (Table 4). On the contrary, drought stress significantly increased root length in mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing soybean plants as compared to no stress and salinity stress conditions.

**Enhanced tolerance to drought and salinity stresses in plants with higher GmFAD3A expression**

Overexpression of FAD3 or FAD8 in tobacco plants is known to increase osmotic stress tolerance (Zhang et al. 2005). This investigation impelled us to investigate impact of GmFAD3A overexpression on drought and salinity stress tolerance in soybean plants. Mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing soybean plants were subjected to drought and salinity stresses. With increasing water deficit, mock-inoculated, vector-infected and FAD3-silenced plants started wilting following by drooping of leaves, although very less drooping was observed in GmFAD3A-overexpressing plants when subjected to drought stress conditions (Fig. 3a). Mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing soybean plants were watered with 100–150 mM NaCl solution in pots filled with soilrite (mixture of peat moss, perlite and vermiculite) for assessing impact of salinity stress. Mock-inoculated, vector-infected and FAD3-silenced plants started exhibiting leaf scorching and this scorching progressively led to leaf necrosis with increasing exposure to salt stress within 4 days of salinity stress (150 mM NaCl). Interestingly, GmFAD3A-overexpressing plants did not develop any leaf scorching (Fig. 3b). Mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing soybean plants grew well under no stress conditions (Fig. 3c). Mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing soybean plants were also analyzed for root system architecture. It was found that GmFAD3A-overexpressing soybean plants, cv. Essex, revealed efficient rooting system in terms of longer roots and higher biomass (dry weight) as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants (Fig. 3d).

**Higher chlorophyll content, SPAD value, maximum Quantum Yield (Qmax) and relative water content under drought and salinity stress conditions in GmFAD3A-overexpressing soybean plants**

Chlorophyll content was significantly higher in GmFAD3A-overexpressing soybean plants as compared to mock-inoculated, vector-infected and FAD3-silenced under no stress as well as under drought and salinity stress conditions (150 mM NaCl) (Fig. 4a). In drought and salinity stress condition,

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**Table 1** Fatty acid profile in mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing soybean plants, cv Essex

| Plants used     | Tissue used | Fatty acid content (mol%) | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 |
|-----------------|-------------|---------------------------|------|------|------|------|------|
| Mock-inoculated | Leaf        | 17.4 ± 0.5                | 4.1  | 0.5  | 2.5  | 0.2  | 16.8 | 1.8  | 42.5 ± 1.5 |
|                 | Immature seed| 11.5 ± 1.1                | 4.8  | 0.4  | 9.2  | 0.7  | 31.1 | 0.7  | 26.5 ± 1.5 |
|                 | Mature seed | 15.1 ± 0.5                | 3.5  | 0.2  | 16.5 | 0.4  | 58.5 | 3.6  | 10.9 ± 1.5 |
| Vector-infected | Leaf        | 16.5 ± 1.2                | 4.5  | 0.5  | 2.8  | 0.3  | 16.2 | 1.3  | 41.5 ± 1.5 |
|                 | Immature seed| 11.0 ± 0.6                | 4.0  | 0.3  | 8.8  | 0.8  | 32.1 | 0.7  | 28.1 ± 1.5 |
|                 | Mature seed | 14.3 ± 1.1                | 2.8  | 0.6  | 15.6 | 0.5  | 56.5 | 1.8  | 11.2 ± 2.5 |
| FAD3-silenced   | Leaf        | 18.5 ± 0.3                | 5.5  | 0.2  | 5.4  | 0.1  | 38.0 | 1.4  | 31.2 ± 1.7 |
|                 | Immature seed| 12.4 ± 1.2                | 4.9  | 0.5  | 8.5  | 0.6  | 45.5 | 1.9  | 19.1 ± 2.1 |
|                 | Mature seed | 16.3 ± 1.4                | 2.5  | 0.7  | 15.1 | 0.8  | 55.5 | 1.5  | 10.2 ± 2.3 |
| GmFAD3A-overexpressing | Leaf | 17.8 ± 0.5 | 4.7  | 0.4  | 4.1  | 0.2  | 12.0 | 1.6  | 69.5 ± 2.7 |
|                 | Immature seed| 10.4 ± 0.5                | 4.8  | 0.3  | 9.5  | 0.3  | 20.5 | 1.6  | 39.5 ± 2.8 |
|                 | Mature seed | 15.9 ± 0.8                | 3.1  | 0.3  | 16.1 | 0.3  | 55.5 | 2.5  | 11.5 ± 2.5 |
| LSD < 0.05      | Leaf        | 2.5 m                     | 1.5 m| 1.5 m| 1.5 m| 1.5 m| 1.5 m| 1.5 m| 3.9 m| 1.5 m |
|                 | Immature seed| 1.5 m                     | 0.9 m| 1.7 m| 1.7 m| 1.7 m| 2.4 m| 2.4 m| 3.9 m| 1.5 m |
|                 | Mature seed | 2.5 m                     | 1.3 m| 1.7 m| 1.7 m| 1.7 m| 3.9 m| 1.5 m| 1.5 m| 1.5 m |

Fatty acid content ± SE. Experiments were repeated three times. n = 3

The LSD values at P < 0.05 were applied to compare the significant differences among the mean values for fatty acids contents in leaf, immature seed and mature seed

At P < 0.05, *indicate significant and ns non-significant differences between the means of fatty acid content in leaf, immature seed and mature seed of different plants types (mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-Overexpressing soybean plants)
reduction in chlorophyll content was less in *GmFAD3A*-overexpressing plants as compared to mock-inoculated, vector-infected and FAD3-silenced. When *GmFAD3A*-overexpressing plants were subjected to 150 mM NaCl, chlorophyll content decreased from 3.07 ± 0.18 to 2.19 ± 0.07 mg/g FW. On the other hand, when mock-inoculated, vector-infected, and FAD3-silenced plants were treated with 150 mM of NaCl for 4 days, chlorophyll content decreased from 2.14 ± 0.14 to 0.99 ± 0.07 mg/g FW, 1.89 ± 0.12–0.76 ± 0.07 mg/g FW, 2.16 ± 0.13–1.30 ± 0.09 mg/g FW, respectively. Upon exposure to drought stress, the chlorophyll content decreased from 2.14 ± 0.14 to 1.37 ± 0.25 mg/g FW, 1.89 ± 0.12–1.04 ± 0.24 mg/g FW, 2.16 ± 0.13–1.64 ± 0.17 mg/g FW, respectively. 

### Table 2 Yield-related attributes in mock-inoculated, vector-infected, FAD3-silenced and *GmFAD3A*-overexpressing soybean plants

| Soybean genotypes | Plant types               | Number of seeds/plant | Seed width (mm) | Seed length (mm) | 100 seed weight (g) |
|-------------------|---------------------------|-----------------------|-----------------|------------------|---------------------|
| Essex             | Mock-inoculated           | 203 ± 1.72            | 4.0 ± 0.24      | 5.0 ± 0.37       | 12.0 ± 0.62         |
|                   | Vector-infected           | 200 ± 1.51            | 4.2 ± 0.11      | 5.2 ± 0.48       | 12.5 ± 0.54         |
|                   | FAD3-silenced             | 198 ± 1.43            | 5.9 ± 0.30      | 6.8 ± 0.54       | 17.0 ± 0.43         |
|                   | *GmFAD3A*-overexpressing | 203 ± 0.61            | 4.1 ± 0.21      | 5.1 ± 0.21       | 12.7 ± 0.48         |
| LSD < 0.05        | 0.55                      | 0.95                  | 1.06            |                  |                     |
| Harosoy           | Mock-inoculated           | 212 ± 1.84            | 4.1 ± 0.42      | 5.5 ± 0.51       | 12.2 ± 0.57         |
|                   | Vector-infected           | 210 ± 1.57            | 4.3 ± 0.31      | 5.7 ± 0.47       | 12.6 ± 0.35         |
|                   | FAD3-silenced             | 205 ± 0.94            | 6.2 ± 0.54      | 7.0 ± 0.64       | 17.5 ± 0.94         |
|                   | *GmFAD3A*-overexpressing  | 212 ± 1.04            | 4.2 ± 0.31      | 5.4 ± 0.34       | 12.8 ± 0.45         |
| LSD < 0.05        | 0.62                      | 0.92                  | 1.26            |                  |                     |
| Williams          | Mock-inoculated           | 255 ± 1.92            | 4.2 ± 0.37      | 4.9 ± 0.22       | 11.5 ± 0.54         |
|                   | Vector-infected           | 250 ± 2.01            | 4.3 ± 0.24      | 5.1 ± 0.11       | 12.1 ± 0.70         |
|                   | FAD3-silenced             | 247 ± 1.52            | 6.5 ± 0.41      | 6.7 ± 0.34       | 16.5 ± 1.15         |
|                   | *GmFAD3A*-overexpressing  | 255 ± 1.34            | 4.1 ± 0.30      | 5.2 ± 0.27       | 12.5 ± 0.54         |
| LSD < 0.05        | 0.65                      | 0.88                  | 1.38            |                  |                     |
| NRC-37            | Mock-inoculated           | 155 ± 1.22            | 4.0 ± 0.45      | 5.0 ± 0.52       | 11.0 ± 0.54         |
|                   | Vector-infected           | 150 ± 1.51            | 4.2 ± 0.51      | 5.3 ± 0.32       | 11.5 ± 0.43         |
|                   | FAD3-silenced             | 145 ± 0.83            | 6.0 ± 0.32      | 6.9 ± 0.25       | 17.0 ± 0.81         |
|                   | *GmFAD3A*-overexpressing  | 155 ± 0.75            | 4.3 ± 0.25      | 5.1 ± 0.42       | 11.8 ± 0.47         |
| LSD < 0.05        | 0.75                      | 0.82                  | 1.42            |                  |                     |
| JS-335            | Mock-inoculated           | 156 ± 1.54            | 4.1 ± 0.21      | 4.8 ± 0.12       | 12.0 ± 0.78         |
|                   | Vector-infected           | 152 ± 1.25            | 4.3 ± 0.35      | 5.2 ± 0.23       | 12.5 ± 0.57         |
|                   | FAD3-silenced             | 158 ± 0.96            | 5.9 ± 0.48      | 6.8 ± 0.36       | 16.5 ± 0.94         |
|                   | *GmFAD3A*-overexpressing  | 163 ± 1.31            | 4.5 ± 0.56      | 5.1 ± 0.46       | 12.8 ± 0.45         |
| LSD < 0.05        | 0.74                      | 0.95                  | 1.34            |                  |                     |

Mean ± SE. Experiments were repeated three times. n = 3

*ns* non significant

### Table 3 Soilrite moisture content after withholding watering for different time periods (0–4 days) of mock-inoculated, vector-infected, FAD3-silenced and *GmFAD3A*-overexpressing soybean plants

| Plant types               | Moisture content in soilrite after withholding watering (%) |
|---------------------------|----------------------------------------------------------|
|                           | 0 day          | 1 day          | 2 day          | 3 day          | 4 day          |
| Mock-inoculated           | 30.2 ± 0.69    | 26.9 ± 0.11    | 23.2 ± 0.22    | 20.0 ± 0.19    | 16.1 ± 0.11    |
| Vector-infected           | 30.8 ± 0.65    | 27.4 ± 0.56    | 23.9 ± 0.30    | 20.2 ± 0.40    | 16.4 ± 0.22    |
| FAD3-silenced             | 29.3 ± 0.58    | 26.0 ± 0.51    | 22.3 ± 0.77    | 19.4 ± 0.56    | 15.6 ± 0.11    |
| *GmFAD3A*-overexpressing  | 29.8 ± 0.60    | 25.9 ± 0.59    | 22.1 ± 0.29    | 19.0 ± 0.19    | 16.0 ± 0.20    |
| LSD < 0.05                | ns             | ns             | ns             | ns             | ns             |

Mean ± SE. Experiments were repeated three times. n = 3

*ns* non significant
mock-inoculated, vector-infected and FAD3-silenced soybean plants. However, in case of GmFAD3A-overexpressing plants, chlorophyll content reduced from 3.07 ± 0.18 to 2.41 ± 0.22 mg/g FW under drought stress conditions.

GmFAD3A-overexpressing plants exhibited increase in SPAD value as compared to mock-inoculated, vector-infected plants and FAD3-silenced plants under no stress, drought and salinity stress conditions (Fig. 4b). The SPAD value decreased markedly in mock-inoculated, vector-infected, and FAD3-silenced plants as compared to GmFAD3A-overexpressing plants under drought and salinity stress conditions. Under drought stress conditions, the SPAD value decreased from 48.66 ± 3.30 to 38.33 ± 3.06, 45.66 ± 4.52–35.22 ± 3.06, 53.55 ± 1.59–41.33 ± 2.11, respectively in mock-inoculated, vector-infected and FAD3-silenced plants, but from 64.34 ± 2.43–52.88 ± 2.18 in GmFAD3A-overexpressing plants. When mock-inoculated, vector-infected and FAD3-silenced plants were exposed to 150 mM NaCl salinity stress, the SPAD value decreased from 48.66 ± 3.30 to 33.22 ± 3.03, 45.66 ± 4.52–31.00 ± 2.30, 53.55 ± 1.59–37.55 ± 1.40, respectively. By contrast, SPAD value in GmFAD3A-overexpressing plants decreased from 64.34 ± 2.43 to 49.55 ± 1.19, when subjected to 150 mM NaCl salinity stress.

Maximum quantum yield (Qmax) in terms of Fv/Fm value indicates efficiency of photosystem II which is adversely affected under drought and salinity stress condition. In the present study, Fv/Fm values was higher in GmFAD3A-overexpressing plants as compared to mock-inoculated, vector-infected and FAD3-silenced plants under no stress, drought and salinity stress condition (Fig. 4c). Maximum quantum yield (Fv/Fm) value markedly decreased in mock-inoculated, vector-infected and FAD3-silenced plants as compared to GmFAD3A-overexpressing plants under drought and salinity stress conditions (Fig. 4c). Mock-inoculated, vector-infected, and FAD3-silenced plants exposed to 150 mM NaCl salt stress recorded a reduction in the Fv/Fm values from 0.76 ± 0.03 to 0.45 ± 0.01, 0.72 ± 0.02–0.38 ± 0.02, 0.75 ± 0.02–0.48 ± 0.02, respectively. In contrast, Fv/Fm values in GmFAD3A-overexpressing plants under salinity stress decreased from 0.89 ± 0.02 to 0.64 ± 0.03. Upon exposure to drought stress, the Fv/Fm value decreased from 0.76 ± 0.03 to 0.45 ± 0.01, 0.72 ± 0.03–0.42 ± 0.01, 0.75 ± 0.02–0.51 ± 0.03, respectively in mock-inoculated, vector-infected and FAD3-silenced plants, but from 0.89 ± 0.02 to 0.69 ± 0.03 mg/g-FW in GmFAD3A-overexpressing plants.

Under well-water (no stress), drought and salinity stress condition, GmFAD3A-overexpressing plants exhibited higher RWC as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants (Fig. 4d). It was observed that decrease in RWC was less in GmFAD3A-overexpressing plants as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants under drought and salinity stress conditions. Under drought stress conditions, the RWC decreased from 72 ± 2.04 to 56.89 ± 4.31, 67.11 ± 2.12–51.44 ± 2.55, 74.00 ± 2.56–57.89 ± 2.18, respectively in mock-inoculated, vector-infected and FAD3-silenced plants, but from 88.78 ± 2.38 to 74.22 ± 1.92 in GmFAD3A-overexpressing plants. When mock-inoculated, vector-infected and FAD3-silenced plants were exposed to salinity stress, the RWC decreased from

### Table 4

| Plant types          | Treatment         | Plant height(cm) | Root length(cm) | Shoot dry weight (g) | Root dry weight (g) |
|----------------------|-------------------|------------------|-----------------|----------------------|---------------------|
| Mock-inoculated      | No stress         | 37.50 ± 0.59     | 19.81 ± 0.56    | 1.60 ± 0.07          | 0.48 ± 0.02         |
|                      | Drought stress    | 32.02 ± 0.47     | 21.12 ± 0.65    | 1.28 ± 0.06          | 0.35 ± 0.03         |
|                      | Salinity stress   | 34.44 ± 0.50     | 18.56 ± 0.52    | 1.43 ± 0.05          | 0.40 ± 0.02         |
| Vector-infected      | No stress         | 36.33 ± 0.55     | 18.22 ± 0.60    | 1.52 ± 0.06          | 0.42 ± 0.03         |
|                      | Drought stress    | 30.50 ± 0.60     | 21.03 ± 0.60    | 1.19 ± 0.05          | 0.32 ± 0.04         |
|                      | Salinity stress   | 33.67 ± 0.53     | 17.33 ± 0.52    | 1.26 ± 0.07          | 0.37 ± 0.03         |
| FAD3-silenced        | No stress         | 37.06 ± 0.63     | 19.78 ± 0.75    | 1.64 ± 0.06          | 0.52 ± 0.02         |
|                      | Drought stress    | 31.72 ± 0.52     | 22.22 ± 0.55    | 1.25 ± 0.06          | 0.38 ± 0.03         |
|                      | Salinity stress   | 34.36 ± 0.65     | 18.03 ± 0.52    | 1.40 ± 0.06          | 0.42 ± 0.02         |
| GmFAD3A-Overexpressing| No stress         | 36.50 ± 0.58     | 24.29 ± 0.62    | 1.72 ± 0.08          | 0.72 ± 0.03         |
|                      | Drought stress    | 34.33 ± 0.62     | 26.06 ± 0.61    | 1.55 ± 0.08          | 0.60 ± 0.04         |
|                      | Salinity stress   | 35.42 ± 0.61     | 23.81 ± 0.52    | 1.63 ± 0.06          | 0.65 ± 0.04         |
| LSD <0.05            | No stress         | 2.50             | 1.35            | 0.12                 | 0.11                |
|                      | Drought stress    | 2.25             | 1.53            | 0.08                 | 0.09                |
|                      | Salinity stress   | 1.23             | 1.65            | 0.10                 | 0.10                |

Mean ± SE. Experiments were repeated three times. n = 3
GmFAD3A-overexpressing plants have lower canopy temperature

GmFAD3A-overexpressing plants showed lower canopy temperature as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants under no stress and drought stress conditions (Fig. 5a). Under drought stress condition, canopy temperature markedly increased in mock-inoculated, vector-infected and FAD3-silenced plants as compared to GmFAD3A-overexpressing plants (Fig. 5a). The canopy temperature under drought stress condition increased from 27.78 ± 0.92 to 31.68 ± 0.72 °C, 28.43 ± 0.77–32.33 ± 0.84 °C, 26.9 ± 0.84–29.03 ± 0.57 °C, respectively in mock-inoculated, vector-infected, FAD3-silenced plants. However, the canopy temperature raised from 23.91 ± 0.89 to 26.15 ± 0.79 °C in GmFAD3A-overexpressing soybean plants. The results showed that under drought stress condition, GmFAD3A-overexpressing plants maintained comparatively cooler canopy as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants.
GmFAD3A-overexpressing soybean plants have higher transpiration rate and stomatal conductance

Transpiration controls water absorption from roots and regulates water status of plants. The GmFAD3A-overexpressing plants exhibited significantly higher transpiration rate compared to mock-inoculated, vector-infected and FAD3-silenced plants under drought and salt stress conditions (Fig. 5b). When mock-inoculated, vector-infected, and FAD3-silenced plants were subjected to salinity stress (150 mM NaCl), transpiration rate decreased from 2.02 ± 0.21 to 1.00 ± 0.08 mmol m⁻² s⁻¹, 1.73 ± 0.12–0.87 ± 0.06 mmol m⁻² s⁻¹, 2.44 ± 0.83–1.37 ± 0.03 mmol m⁻² s⁻¹, respectively. In contrast, when GmFAD3A-overexpressing plants were subjected to salinity stress, the transpiration rate decreased from 3.5 ± 0.21 to 2.10 ± 0.12 mmol m⁻² s⁻¹.

Under drought stress condition, the transpiration rate decreased from 2.02 ± 0.21 to 1.43 ± 0.11 mmol m⁻² s⁻¹, 1.73 ± 0.12–1.24 ± 0.11 mmol m⁻² s⁻¹, 2.44 ± 0.08–1.68 ± 0.22 mmol m⁻² s⁻¹, respectively in mock-inoculated, vector-infected and FAD3-silenced plants. However, in GmFAD3A-overexpressing plants, transpiration rate decreased from 3.5 ± 0.21 to 2.41 ± 0.25 mmol m⁻² s⁻¹.

Under no stress and drought and salinity stress conditions, GmFAD3A-overexpressing plants exhibited higher stomatal conductance as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants (Fig. 5c). Stomatal conductance in mock-inoculated, vector-infected and FAD3-silenced plants under salinity stress decreased from 0.21 ± 0.01 to 0.12 ± 0.005 mmol m⁻² s⁻¹, 0.18 ± 0.01–0.11 ± 0.01 mmol m⁻² s⁻¹, 0.23 ± 0.005 to 0.14 ± 0.02 mmol m⁻² s⁻¹, respectively. In contrast, stomatal conductance in GmFAD3A-overexpressing plants decreased from 0.32 ± 0.05 to 0.26 ± 0.01 mmol m⁻² s⁻¹. Stomatal conductance under drought stress condition reduced from 0.21 ± 0.01 to 0.15 ± 0.01 mmol m⁻² s⁻¹, 0.18 ± 0.01–0.13 ± 0.01 mmol m⁻² s⁻¹, 0.23 ± 0.005–0.17 ± 0.01 mmol m⁻² s⁻¹, respectively in mock-inoculated, vector-infected and
FAD3-silenced plants, but from 0.32 ± 0.005–0.26 ± 0.01 mmol m⁻² s⁻¹ in GmFAD3A-overexpressing 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 ($P<0.05$). Asterisk denotes significant difference.

GmFAD3A-overexpressing plants accumulate higher levels of jasmonic acid under drought and salinity stress conditions

Jasmonic acid and its metabolites collectively known as jasmonates and play an important role in plant development and biotic and abiotic stress responses (Wasternack et al. 2013). The FAD3 mediates unsaturation of linoleic acid to produce α-linolenic acid. Since α-linolenic acid is a precursor for JA biosynthesis, the JA levels were quantified in mock-inoculated, vector-infected, FAD3-silenced as well as GmFAD3A-overexpressing plants under no stress and also under drought and salinity stress conditions. The JA levels were not significantly altered under no stress, drought and salinity stress conditions in mock, vector-infected and FAD3-silenced soybean plant. The GmFAD3A-overexpressing plants showed approximately three-fold and six-fold higher levels of JA under no stress, and drought and salinity stress conditions as compared to vector-infected soybean plants, respectively (Fig. 6a). These results indicate that JA plays an important role in drought and salinity stress tolerance in soybean.

Increase in proline level in GmFAD3A-overexpressing plants under drought and salinity stress conditions

Earlier reports demonstrated an increase in proline content in response to drought and salinity stress (Trovato et al. 2008; Goel et al. 2010, 2011). These finding prompted us to measure proline level in mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing plants under no stress, drought and salinity stress conditions. The level of proline accumulated in GmFAD3A-overexpressing plants under drought and a salinity stress condition were much higher than that in mock-inoculated, vector-infected and FAD3-silenced soybean plants (Fig. 6b). Under no stress condition, the proline
content 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, FAD3-silenced and GmFAD3A-overexpressing plants. Under drought stress conditions, the proline content increased from 2.17 ± 0.35 to 3.04 ± 0.45 mg/g FW, 1.94 ± 0.43–3.43 ± 0.48 mg/g FW, 2.61 ± 0.54–4.01 ± 0.54 mg/g FW, 2.77 ± 0.53–6.79 ± 0.48 mg/g FW, respectively in mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing plants. When mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing plants were subjected to salinity stress, the proline content increased from 2.17 ± 0.35 to 4.1 ± 0.48 mg/g FW, 1.94 ± 0.43–4.3 ± 0.55 mg/g FW, 2.61 ± 0.54–5.5 ± 0.65 mg/g FW, 2.77 ± 0.53–7.5 ± 0.55 mg/g FW, respectively.

Expression of GmWRKY transcription factors in GmFAD3A-overexpressing plants under drought and salinity stresses

Differential tolerance to abiotic stress was achieved in Arabidopsis when transformed with GmWRKY transcription factors (Zhou et al. 2008; Li et al. 2020). This prompted us to study the expression of GmWRKY transcription factors like GmWRKY13, GmWRKY19, GmWRKY21, GmWRKY39, GmWRKY54 and GmWRKY62 in mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing plants under no stress, drought and salinity stress conditions. Higher expression of GmWRKY54 transcription factor was detected in GmFAD3A-overexpressing plants as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants under drought and salinity stress condition (Fig. 7a). Interestingly, in non-stressed plants, expression of GmWRKY54 transcription factor did not change in mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing plants (Fig. 7a). Transcription factors such as GmWRKY13, GmWRKY19, GmWRKY21 transcription factors were induced under drought and salinity stress conditions in mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing soybean plants and induction was almost similar, but these GmWRKY transcription factors were not induced under no stress conditions (Figs. 7b–d). Mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing plants were also evaluated for expression of GmWRKY39 and GmWRKY62 transcription factors and it was observed that these GmWRKY transcription factors were not induced under no stress, drought and salinity stress conditions (Figs. 7e–f).

Discussion

Abiotic stresses have a profound impact on almost all aspects including growth, development and reproduction in plants (Devireddy et al. 2021; Rane et al. 2021a, b, c). Therefore, it is crucial to understand abiotic stress responses in plants in order to enhance abiotic 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 abiotic stress and its stabilization is vital for survival of the plant (Zhang et al. 2005; Shi et al. 2008). Membrane stability and its integrity maintenance is
largely affected by lipid content and fatty acid desaturation (Mikami and Murata 2003; Shi et al. 2008). Hence, fatty acid desaturation by fatty acid desaturases and increase in linolenic acid are considered critical factors for tolerance of plants to multiple abiotic stresses (Upchurch 2008). The fatty acid desaturase 3 (FAD3) is known to mediate conversion of linoleic to α-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 GmFAD3A gene were manipulated in soybean by overexpression as well as silencing of FAD3 using a BPMV-based viral vector. Overexpression of GmFAD3A gene was confirmed by RT-qPCR and RNA blot analyses. Overexpression of GmFAD3A employing BPMV-based viral vector resulted very high level of α-linolenic acid which in turn resulted drought and salinity stress tolerance. 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) isolated a microsomal ω-3 FAD gene from a cryophyte (Chorispora bungeana) (CbFAD3), exhibiting a high identity to Arabidopsis FAD3, and the functionality of CbFAD3 were analyzed in C. bungeana suspension-cultured cells, yeast cells, and also in transgenic tobacco plants expressing CbFAD3 under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Shi et al. (2018) reported that overexpression of CbFAD3 increased linolenic acid (C18:3) in both leaves and roots, which in turn enhanced plant tolerance to drought and salt stresses in transgenic tobacco and correlated it with activation of reactive oxygen species scavenging system, plasma membrane Ca^{2+}-ATPase, stress-induced Ca^{2+} signaling and regulation of stress responsive genes. In another study, yeast transformed with ω-6 desaturases from sunflower had increased salt tolerance (Upchurch 2008). Recently, Rane et al. (2021a, b, c) studied implications of reactive oxygen species and antioxidative system with respect to effective use of water in crop plants.

In the present study, plant growth, shoot and root biomass (dry weight) were reduced in mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing soybean plants under drought and salinity stress conditions. However, plant height, shoot and root biomass (dry weight)
were less affected in *GmFAD3A*-overexpressing soybean plants as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants under no stress conditions. In the present study, longer and efficient rooting system and higher root biomass were observed in *GmFAD3A*-overexpressing soybean plants as compared to mock-inoculated, vector-infected, FAD3-silenced soybean plants under no stress conditions. Under drought stress condition, the root length increased in mock-inoculated, vector-infected, FAD3-silenced and *GmFAD3A*-overexpressing soybean plants as compared to no stress and salinity stress conditions. The *GmFAD3A*-overexpressing soybean plants had a larger root system that most probably optimized water uptake. The longer root structure with high biomass enables *GmFAD3A*-overexpressing soybean plant to tolerate drought and salinity stress tolerance by up taking water more efficiently and ultimately maintaining higher water status as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants. Drought reduces water potential in soil, hence decreasing the amount of water available to plant roots. This low availability of soil moisture causes decrease in nutrient transport towards roots. Water deficit condition impaired active transport and membrane permeability and reduction of transpiration rate which resulted decrease in the nutrients absorption efficiency of roots (Kramer and Boyer 1995). Hence, due to water deficit condition and nutritional imbalance, plant growth is reduced (Hu et al. 2006). Salinity stress also results nutritional imbalance and ion toxicity due to higher ionic ratios in addition to reducing water uptake by roots due to low osmotic potential. Therefore, reduction in plant growth under saline conditions might occur either due to reduced water availability or the direct toxic effects of sodium chloride (Munns and Tester 2008). Also, reduction in shoot and root dry weight may be a result of a restricted hydrolysis of food reserves and their translocation to shoots. Root system architecture, particularly longer root length plays an important role in maximizing the ability of plants to take water and nutrients for plant growth (Fenta et al. 2014; Robbins and Dinneny 2018). In the present study, root length was longer under drought condition as compared to no stress that enables efficient water and nutrition uptake. Although salinity stress showed no effect on root length elongation.

In the present investigation, we reported that higher expression of *GmFAD3A* led to enhanced drought and salinity stress tolerance in soybean, while FAD3-silenced plants were vulnerable to drought as well as salinity stresses. These results may be explained based on changes occurred at morphological, physiological, biochemical and molecular levels under non-stress, drought and salinity stress conditions. We observed that *GmFAD3A*-overexpressing soybean plants showed significantly higher chlorophyll content, maximum quantum yield (Fv/Fm), and RWC as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants under no stress as well as under drought and salinity stress conditions. Less decrease in chlorophyll content and leaf SPAD value were observed in *GmFAD3A*-overexpressing plants as compared to mock-inoculated, vector-infected, FAD3-silenced plants under drought and salinity stress conditions. The ability of *GmFAD3A*-overexpressing 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. Maximum quantum yield (Qmax) in terms of Fv/Fm value, which indicates photochemical efficiency varies with severity of drought and decreased drastically during prolonged drought stress (Zivcak et al. 2008; Rane et al. 2019; Rane et al. 2021a, b, c). Kumar et al. (2017) reported that photosynthetic efficiency in soybean was closely associated with canopy greenness reflected by higher chlorophyll content. Markedly 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, FAD3-silenced plants than that of *GmFAD3A*-overexpressing 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. The *GmFAD3A*-overexpressing plants showed relatively higher RWC compared to mock-inoculated, vector-infected and FAD3-silenced plants under no stress, drought and salinity stress condition. It was observed that RWC decreased markedly in mock-inoculated, vector-infected and FAD3-silenced plants as compared to *GmFAD3A*-overexpressing plants under drought and salinity stress condition. Drought and salinity stress induced reduction in the relative water content indicated a decrease in turgor that resulted in limited water availability for cellular process in mock-inoculated, vector-infected and FAD3-silenced plants as compared to *GmFAD3A*-overexpressing soybean plants.

Canopy temperature was found lower in *GmFAD3A*-overexpressing plants as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants under no stress, drought and salinity 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; Taria et al. 2019). Lower canopy temperature could be due to higher stomatal conductance and transpiration rate in *GmFAD3A*-overexpressing plants as compared to mock-inoculated, vector-infected...
and FAD3-silenced plants under drought and salinity stress conditions.

The GmFAD3A-overexpressing soybean plants had higher JA levels under drought and salinity stress conditions as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants. This result suggested important role of jasmonate in coping with drought and salinity stresses in GmFAD3A-overexpressing soybean plants. 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 in tobacco by improving Fv/Fm, alleviating degradation of chlorophyll and protecting PS-II under drought stress (Wei-Wei et al. 2011), in wheat by increasing photosynthesis rate, delayed senescence and improving water status (Ma et al. 2014). Salinity stress tolerance was also improved by exogenous application of MeJA in several plants such as in tomato (Enteshari and 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 and/or a hexadecanoid pathway that uses oleic acid as a precursor.

Proline is an important organic compatible solute, which protects plants against free radical-induced damage under stress condition. Many plants accumulate proline in response to abiotic stresses such as drought and salinity (Trovato et al. 2008). In the present study, it was observed that drought and salinity stresses resulted in significant increase in proline content in GmFAD3A-overexpressing plants as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants. Rice roots exposed to NaCl stress resulted in accumulation of proline with increasing NaCl concentrations (Morant et al. 2004). Increase in proline content under drought and salinity stress might be due to activation of proline syntheses from glutamate or decrease in its utilization during protein syntheses. Gad (2005) reported that proline may be the major source for energy and nitrogen during metabolism after stress and accumulated proline supplies that energy for growth and survival, thereby enhancing drought and salinity stress tolerance.

In the present study, GmFAD3A-overexpressing plants exhibited tolerance to drought and salinity stress and higher expression of GmWRKY54 transcription factor as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants under drought and salinity stress conditions. Earlier, GmWRKY54-overexpressing transgenic Arabidopsis plants were evaluated under abiotic stress (Zhou et al. 2008). Over-expression of GmWRKY54 resulted in enhanced tolerance to drought and salt stress in transgenic Arabidopsis plants. Expression of GmWRKY13, GmWRKY19 and GmWRKY21 transcription factors was higher under drought and salinity stress condition as compared to no stress condition in mock-inoculated, vector infected, FAD3-silenced and GmFAD3A-overexpressing soybean plants. But, expression of GmWRKY13, GmWRKY19 and GmWRKY21 transcription factors was not significantly different in mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing soybean plants under drought and salinity stress conditions. When mock-inoculated, vector-infected, FAD3-silenced and GmFAD3A-overexpressing plants were evaluated for expression of GmWRKY39 and GmWRKY62, there were no significant change in expression of these transcription factors under no stress, drought and salinity stress conditions. Earlier, Zhou et al. (2008) also reported differential expression of GmWRKY like transcription factors in transgenic Arabidopsis under drought and salt stress conditions. The JA-responsive TFs like WRKY regulate the expression of many genes associated with growth and development of plants, and especially the responses and adaptation of plants to the environmental stress. 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 (Finatno et al. 2018). Zhou et al. (2008) reported differential 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 CORONATINEINSENSITIVE1 (COI1) and forms the Skp1/Cullin/F-box (SCFCOI1) complexes. 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). 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).

Earlier, Shi et al. (2018) explained tolerance to drought and salinity stresses in transgenic tobacco plants overexpressing CbFAD3 based on an integrated regulation that
involves increase in membrane stability, photosynthetic capacity and the expression of stress-responsive genes affected by C18:3, Ca^{2+}, or ROS. In the present study, protection of GmFAD3A-overexpressing soybean plants against drought and salinity stresses was observed in the investigation of morphological, physiological, biochemical and molecular changes. The enhanced tolerance of the GmFAD3A-overexpressing 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 and increased level of JA under drought and salinity stress condition as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants. Taken together, enhanced drought and salinity stress tolerance in GmFAD3A-overexpressing plants may be correlated with linolenic acid (C18:3) induced membrane stabilization and the increased expression of stress-responsive genes such as GmWRKY54.

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**Data availability** Enquiries about data availability should be directed to the authors.

**Declarations**

**Conflict of interest** Authors declare no competing interests.

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