Improving nutritional quality and fungal tolerance in soya bean and grass pea by expressing an oxalate decarboxylase

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Summary
Soya bean (Glycine max) and grass pea (Lathyrus sativus) seeds are important sources of dietary proteins; however, they also contain antinutritional metabolite oxalic acid (OA). Excess dietary intake of OA leads to nephrolithiasis due to the formation of calcium oxalate crystals in kidneys. Besides, OA is also a known precursor of β-N-oxalyl-L-α,β-diaminopropionic acid (β-ODAP), a neurotoxin found in grass pea. Here, we report the reduction in OA level in soya bean (up to 73%) and grass pea (up to 75%) seeds by constitutive and/or seed-specific expression of an oxalate-degrading enzyme, oxalate decarboxylase (FvOXDC) of Flammulina velutipes. In addition, β-ODAP level of grass pea seeds was also reduced up to 73%. Reduced OA content was interrelated with the associated increase in seeds micronutrients such as calcium, iron and zinc. Moreover, constitutive expression of FvOXDC led to improved tolerance to the fungal pathogen Sclerotinia sclerotiorum that requires OA during host colonization. Importantly, FvOXDC-expressing soya bean and grass pea plants were similar to the wild type with respect to the morphology and photosynthetic rates, and seed protein pool remained unaltered as revealed by the comparative proteomic analysis. Taken together, these results demonstrated improved seed quality and tolerance to the fungal pathogen in two important legume crops, by the expression of an oxalate-degrading enzyme.

Keywords: Glycine max, Lathyrus sativus, oxalic acid, oxalate decarboxylase, Sclerotinia resistance.

Introduction
Oxalic acid (OA), a strong dicarboxylic acid, is naturally abundant in fruits, vegetables and seeds (Holmes and Kennedy, 2000; Holmes et al., 1998). At physiological pH, OA forms soluble salts with sodium and potassium; however, OA chelates calcium to form insoluble calcium oxalate, which renders OA clinically important. Because humans cannot degrade ingested OA due to the lack of any biological and/or enzymatic system, the consumption of high OA-containing vegetable diet is considered detrimental to the human health. This leads to hyperoxaluria and destruction of renal tissues because of the precipitation of calcium oxalate in the kidneys, a medical condition known as nephrolithiasis (Robertson and Peacock, 1980; Sidhu et al., 1999).

Legumes constitute the main source of proteins to the majority of human diets. Legume proteins are rich in essential amino acid lysine, which is lacked in cereals. Besides, legumes are the source of a wide array of essential nutrients, low glycaemic carbohydrates, dietary fibre, vitamins and minerals, and cultivation of legumes improves soil health of the agricultural and agroforestry systems. Soya bean (Glycine max) and grass pea (Lathyrus sativus) are legumes with abundant economical and nutritional importance. Soya bean seeds contain around 38%–40% proteins, whereas beans and/or peas contain nearly 20% proteins (Gwtek et al., 2014). Moreover, soya bean is responsible for providing more than half of globally consumed edible oil (SoyStats, 2013, available at www.soystats.com). Soya bean has become indispensible because it provides a wide range of potential advantages such as protein-based biodegradable materials, anticancerous constituents and potential to lower cholesterol (Ko et al., 2013). Such varied uses of soya bean made it a more desired crop, and its global demand is increasing day by day (Deshmukh et al., 2014). Grass pea (Lathyrus sativus) is also an agronomically important crop due to its high protein content, good grain yield, resistance to insects as well as pests. Moreover, it has a potential to tolerate extensive drought and water-logging stresses (Yan et al., 2006; Zambre et al., 2002). This crop is cultivated and consumed in various parts of the world not only for animal feed and fodder but also for human consumption, because it is an inexpensive source of dietary lysine-rich protein for people of low-income food-deficit countries (Barik et al., 2005; Smartt, 1990). However, OA found in soya bean and grass pea seeds is an antinutritional factor (Chai et al., 2004; Yan et al., 2006). Oxalate content of soya bean varies from 0.67 to 3.5 g/100 g of seeds dry weight (Massey et al., 2001). Moreover, OA also serves as a precursor for the biosynthesis of a grass pea neurotoxin, β-N-oxalyl-L-α,β-diaminopropionic acid (β-ODAP) (Xiong et al., 2015). Some of the grass pea germplasms also display a significant variability in...
\[\beta\text{-ODAP content, and these grass peas contain 0.5\%–2.5\% }\beta\text{-ODAP in the seeds (Xiong et al., 2015). Prolonged consumption of }L.\text{ sativus causes neuroathy, which is characterized by spasticity of leg muscles, lower limb paralysis, convulsions and death (Yan et al., 2006).}

Besides, OA is considered as a pathogenesis factor for the devastating phytopathogenic fungus Sclerotinia sclerotiorum which synthesizes and secretes OA during host colonization (Kabbage et al., 2015; Malenčič et al., 2010; Uloth et al., 2015). Sclerotinia sclerotiorum, a necrotrophic fungal pathogen, induces white mold of more than 400 plant species, including important crops such as cotton, tomato, sunflower, grass pea and soya bean (Heller and Witt-Geiges, 2013). The pathogenicity of S. sclerotiorum is concomitant with OA secretion by fungus, and OA is thought to play a crucial role in infection. The relationship between Sclerotinia pathogenicity and OA secretion might heighten Sclerotinia virulence mostly by three modes of action (Dutton and Evans, 1996). First, fungus secretes several cell wall-degrading enzymes such as polygalacturonase to invade plant tissues. These enzymes work at low pH, and OA might play an important role in providing the low pH environment for these enzymes. Second, the presence of OA brings down pH of the infection site due to its acidic nature, which further leads to weakening of the plant and thereby facilitates the invasion to plant tissue. Thirdly, because Ca\textsuperscript{2+} is an essential component of plant defence system, OA could bind with Ca\textsuperscript{2+} and form calcium oxalate which may compromise the function of Ca\textsuperscript{2+}-dependent defence. Chelation of cell wall Ca\textsuperscript{2+} by OA might not only compromise the function of Ca\textsuperscript{2+}-dependent defence responses but also weaken the plant cell wall (Bateman and Beer, 1965; Cessna et al., 2000; Dutton and Evans, 1996). This further provides a platform for fungal degradative enzymes to assist the fungal infection (Lumsden, 1979). Moreover, the accumulation of high level of OA during fungal infection results in wilting of the plants and finally cell death (Kim et al., 2008).

Oxalic acid can be biodegraded via two pathways, that is oxidation and decarboxylation. Oxalate oxidase (EC 1.2.3.4), belongs to the germin family of proteins, expressed predominately in higher plants, catalyses the oxygen-dependent oxidation of oxalate to carbon dioxide in a reaction that is coupled with the formation of hydrogen peroxide. Oxalate oxidase activity is found in a wide variety of plants, including barley seedling, roots and leaves, Amaranthus leaves, beet stems and leaves, sorghum leaves and maize, oats, rice and rye (Hurkman et al., 1991; Lathika et al., 1995). Flammulina velutipes oxalate decarboxylase (FvOXDC) is thought to be the best characterized oxalate decarboxylase from the wood-rotting fungus reported till date (Chakraborty et al., 2002). FvOXDC is specific to oxalate, and it catabolizes to formic acid (nontoxic organic acid) and CO\textsubscript{2} in a single step without the requirement of a cofactor. This enzyme is active at low pH which would be helpful as most of the oxalate is localized in plant cell vacuoles, where pH is low. FvOXDC is a hexamer of six identical subunits of 410 kDa molecular mass, and the hexamer is a dimer of trimers (Chakraborty et al., 2002). The Mn\textsuperscript{2+} and OA binding to the active site of FvOXDC plays a key role in enzyme catalysis (Chakraborty et al., 2002).

In this present study, we have developed transgenic soya bean and grass pea lines by constitutive and/or seed-specific expression of FvOXDC with the aim to reduce OA in soya bean and both OA and \(\beta\)-ODAP in grass pea. The reduction of OA in soya bean and grass pea not only improved their seed nutritional quality but also conferred tolerance to S. sclerotiorum.

### Results

#### Expression and stable integration of FvOXDC in transgenic soya bean and grass pea

To reduce the levels of antinutritional OA in soya bean and OA and \(\beta\)-ODAP in grass pea, we have generated transgenic lines for both soya bean and grass pea for CaMV35S-driven constitutive expression of FvOXDC. Moreover, seed-specific expression of FvOXDC was also achieved in soya bean using the seed-specific promoter of soya bean late-embryogenesis-abundant protein (LEA protein; GmPM9). The pSOVA expression plasmid contained FvOXDC under the control of the CaMV35S promoter (Figure 1a). In pSPM9 plasmid, FvOXDC was under the control of seed-specific promoter (Figure 1b). These two FvOXDC constructs (pSOVA and pSPM9) were used to transform soya bean cultivar MACS-57 via Agrobacterium tumefaciens-mediated transformation by following a robust and reproducible transformation protocol involving soya bean cotyledonary nodules as explants (Figure S1). However, for grass pea, embryos were used as explants (Figure S2) to transform cultivar LP-24. FvOXDC gene amplification, following polymerase chain reaction (PCR) using FvOXDC-specific primers and genomic DNA as template, was carried out for the kanamycin resistance and single-copy transgene-integrated T3 (soya bean SOVA lines and PSPM9 lines) and T4 (grass pea SOVA lines) generation transgenic lines (Figure 1bc,i). PCR-amplified DNA fragment of 750 bp was detected in all the kanamycin-resistant transgenic lines, confirming genomic integration of the FvOXDC.

#### FvOXDC protein expression and oxalate decarboxylase activity in transgenic plants

To confirm the expression of FvOXDC in soya bean and grass pea, immunoblot assay was performed using polyclonal anti-FvOXDC antibody. Immunoblot assay using total soluble protein revealed an immunoreactive band of 55 kDa, corresponding to the predicted size of the FvOXDC (Figure 1d,e,j). However, protein band corresponding to the FvOXDC was not detected in the protein extracts of nontransformed (wild-type) plants, confirming specificity of the immunoreactive band. This result confirmed the expression of FvOXDC protein in soya bean and grass pea transgenic lines. Further, to determine the functional expression of FvOXDC in soya bean and grass pea, total protein extracts were examined for the OXDC activity. The OXDC activity was examined by measuring the liberation of 14CO\textsubscript{2} from 14C oxalic acid (Amersham, Little Chalfont, UK). FvOXDC was found to be functionally active with five to sevenfold increase in CO\textsubscript{2} production in transgenic soya bean in pSOVA and pSPM9 FvOXDC-expressing lines, respectively (Figure 1f,g) and two to threefold increase in CO\textsubscript{2} production in transgenic grass pea pSOVA lines (Figure 1k). Moreover, leaves of transgenic and wild type were tested for the OA sensitivity (Foster et al., 2012; Kesarwani et al., 2000). Leaves of FvOXDC-expressing transgenic plants were less chlorotic compared to wild type (Figure 1l). These results confirmed in planta role of FvOXDC in OA degradation.

#### Expression of FvOXDC decreases OA level in soya bean and OA and \(\beta\)-ODAP level in grass pea

FvOXDC-expressing transgenic soya bean and grass pea lines were assayed for seed OA and \(\beta\)-ODAP content and compared with the nontransformed wild-type plants. The analysis revealed
up to 67% reduction in OA level in pSOVA lines (Figure 2a) and up to 73% reduction in pSPM9 lines of soya bean (Figure 3a), whereas OA and JI-ODAP contents of grass pea were reduced up to 75% and 73%, respectively (Figure 4a, b). As FvOXDC-mediated decarboxylation of OA results in the generation of formic acid, the levels of formic acid in the seeds of FvOXDC-

**Figure 1** Molecular analyses of soya bean and grass pea transgenic lines expressing FvOXDC. (a) Schematic representation of the expression plasmid pSOVA, containing FvOXDC-coding sequence under the control of CaMV35S promoter. RB, right border; LB, left border; nptII, neomycin phosphotransferase. (b, c) PCR analysis of transgenic pSOVA soya bean (b) and grass pea (c) lines, for the confirmation of the FvOXDC genomic integration. (d, e) Immunoblot analysis to confirm FvOXDC expression in transgenic pSOVA lines of soya bean (d) and grass pea (e). (f, g) Oxalate decarboxylase activity shown as micromoles of CO2 liberated per minute per milligram of protein at 37 °C. Oxalate decarboxylase activities for the pSOVA lines of soya bean (f) and grass pea (g) are shown. Data represent the values obtained from the means (±SE) of ten leaves per plant for each independent transgenic event and the wild type in triplicate. (h) Schematic representation of the expression plasmid pSPM9, containing FvOXDC-coding sequence under the control of GmPM9 promoter for seed-specific expression. (i) PCR analysis of transgenic pSPM9 soya bean lines for the confirmation of the FvOXDC genomic integration. (j) Immunoblot analysis confirmed FvOXDC expression in transgenic pSPM9 lines of soya bean. (k) Oxalate decarboxylase activity of soya bean plants expressing oxalate decarboxylase in a seed-specific manner, shown as micromoles of CO2 liberated per minute per milligram of protein at 37 °C. Data represent the values obtained from the means (±SE) of ten seeds per plant for each independent transgenic event and the wild type in triplicate. (l) Oxalic acid (OA) sensitivity of the wild type and transgenic plants constitutively expressing FvOXDC was compared. Excised leaves were immediately dipped in the OA solution, and a negative control was kept with wild-type leaf in water (mock).
expressing transgenic lines were also determined. A concomitant increase (70%) in formic acid was observed with the expression of FvOXDC in soya bean transgenic lines (Figures 2b and 3b). Thus, FvOXDC expression was correlated with the reduced level of OA in soya bean and grass pea and reduced β-ODAP level in grass pea.

Reduction in OA content resulted in increased levels of some micronutrients

Micronutrients play a crucial role in human and animal health, and thus, it is desired to generate transgenic plants with higher levels of these components (Gupta et al., 2015; Hefferon, 2015). Because OA is a strong chelator of divalent cations, especially calcium, therefore, we were interested to determine the micronutrient contents in FvOXDC-expressing transgenic soya bean and grass pea seeds. Calcium and other micronutrients such as magnesium, zinc, iron, sodium and potassium were analysed by energy-dispersive X-ray fluorescence analysis (EDXRF). A marked increase in the levels of calcium, magnesium, zinc, iron and manganese in the seeds was recorded. Calcium level increased more than two folds in low-oxalate transgenic pSOVA and pSPM9 lines of soya bean (Figures 2c and 3c). In the seeds of both pSOVA and pSPM9 line, a significant increase in the levels of other nutrients such as Mg, Fe, Zn and Mn was also noticed in comparison with the control seeds (Figures 2d–g and 3d–g). The content of calcium, magnesium, zinc, iron and manganese in grass pea seeds was also significantly increased (Figure 4c–g). These data suggested an increased concentration of micronutrients in the seeds of low-oxalate transgenic soya bean and grass pea.

Reduced OA content led to enhanced tolerance to S. sclerotiorum

Sclerotinia sclerotiorum pathogenicity is associated with OA (Dutton and Evans, 1996). Therefore, we tested the response of transgenic soya bean and grass pea, constitutively expressing FvOXDC, to the S. sclerotiorum infection. Transgenic soya bean and grass pea and wild-type plants were infected with S. sclerotiorum as described in Experimental procedures using mycelia suspension, and the progression of disease symptoms was monitored for different time intervals. Inoculated leaves of transgenic and wild types were considered as infected with...
S. sclerotiorum when the symptoms of tissue browning and lesion formation appeared. Compared to the nontransgenic wild type, FvOXDC-expressing transgenic leaves displayed a substantial delay in the lesion progression (Figure 5a, b). Lesion intensity was also low in transgenic pSOVA lines of both soya bean and grass pea as compared to their corresponding wild-type plants (Figure 5c, d).

Quantitative proteomic analysis revealed unaltered protein profile in the seeds of transgenic plants

Seeds of transgenic soya bean and grass pea were examined for their protein profile using isobaric tags for relative and absolute quantitation (iTRAQ) analysis and compared to the wild type as described in Experimental procedures. To understand protein profile of the transgenic and wild-type seeds, 4 plex iTRAQ labels were used. Proteins of the wild type were labelled with 114 and 115, whereas proteins isolated from transgenic grass pea were labelled with 116 and 117. iTRAQ analysis revealed unaltered protein profiles in the seeds of transgenic and wild-type soya bean and grass pea (Figure S3a, b and Tables S1 and S2).

Transgenic soya bean and grass pea resembled wild-type plants with respect to growth and developmental phenotype and physiology

Transgenic soya bean and grass pea were compared with wild-type plants, either in the primary transgenic populations or in the subsequent generations, and there were no observable differences in phenotype, development or fertility in any of five independent transgenic line of each soya bean (pSOVA, pSPM9) and grass pea (pSOVA) transgenic lines. Transgenic lines were also checked for their field performance by transplanting them in the experimental field alongside wild-type plants and randomly replicated for further generations. Transgenic plants were normal

Figure 3 Biochemical analyses of transgenic soya beans expressing FvOXDC in a seed-specific manner. (a, b) Quantitation of OA and formic acid in soya bean seeds expressing FvOXDC under the control of pSPM9 seed-specific promoter. Levels of calcium (c), magnesium (d), iron (e), zinc (f) and manganese (g) were determined using energy-dispersive X-ray fluorescence spectrophotometry. Data represent the values obtained from the means (±SE) of ten seeds per plant for each independent transgenic event and the wild type in triplicate.

Reduced oxalic acid in fungal tolerant legumes
in vegetative growth, and their morphology was normal as compared to the wild-type plants. Transgenic plants were similar in flowering time, vigour or total yield as observed for wild type. Moreover, expression of \textit{FvOXDC} did not affect the net photosynthetic rates or other physiological parameters, including stomatal conductance and transpiration rate.

**Discussion**

With long exposure of plant breeding techniques and genetically modification through transgenic technology, there has been a substantial success in augmenting the nutritional value of food crops. Efforts have been made to improve the quality and quantity of macronutrients such as protein/amino acids, oils/fats and carbohydrates as well as to increase the bioavailability of micronutrients, vitamins and antioxidants through transgenics and breeding. In soya bean, most of the work was carried out to improve seed protein quality (Cunha et al., 2011; Nishizawa et al., 2010; Qi et al., 2011), oil content (Kajikawa et al., 2008; Li et al., 2010), biotic resistance (Cunha et al., 2010; Dang and Wei, 2007; McLean et al., 2007; Tougou et al., 2007) and abiotic resistance (DeRonde et al., 2004; Valente et al., 2009). However, on the other hand, there have been limited efforts in the management of antinutrients and/or toxins, which are the byproducts of plant metabolism and considered as health hazards to humans and animals. OA is one of the known antinutritional factors, which has negative health concerns, when we consume crops containing high amount of OA, such as tomato, spinach, soya bean and grass pea.

Legumes are very challenging to regenerate and transform because somatic embryogenesis or organogenesis is found to be difficult (lantcheva et al., 2013). Here, in the present study, we have developed an efficient transformation method for both soya bean and grass pea using cotyledonal explants and embryo explants, respectively (Figures 1, S1 and S2). Further, to improve the nutritional quality of soya bean and grass pea by down-

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**Figure 4** Biochemical analyses of transgenic grass pea lines constitutively expressing \textit{FvOXDC}. (a, b) Quantitation of ODAP and OA in grass pea expressing \textit{FvOXDC} in a constitutive manner. Levels of calcium (c), magnesium (d), iron (e), zinc (f) and manganese (g) were determined using energy-dispersive X-ray fluorescence spectrometry in grass pea transgenic lines expressing \textit{FvOXDC} in a constitutive manner. Data represent the values obtained from the means (±SE) of ten seeds per plant for each independent transgenic event and the wild type in triplicate.

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regulating OA, we have developed transgenic plants expressing a well-characterized FvOXDC (Azam et al., 2001, 2002; Chakraborty et al., 2002, 2013; Kesarwani et al., 2000). Our results showed that there was a significant decrease in OA content in both the lines of pSOVA and pSPM9 (Figures 2a and 3a). Transgenic soya bean plants expressing FvOXDC in a seed-specific manner were superior in OA management than the constitutive lines. OA and β-ODAP contents of grass pea were also assayed biochemically, and their contents were found to be reduced (Figure 4a,b). Expression of FvOXDC in soya bean and grass pea not only led to a significant reduction in OA levels but also associated with a substantial increase in calcium, conceivably by down-regulating the content of OA that chelates calcium (Figures 2c, 3c and 4c). We have also found an increase in other elements such as iron, manganese, magnesium and zinc in transgenic lines of soya bean and grass pea (Figures 2d–g, 3d–g and 4d–g). Previous reports suggested that the expression of Ca$^{2+}$/H$^+$ exchangers can alter the sum of all the mineral nutrients (Cheng et al., 2005; Mei et al., 2007). However, in FvOXDC-expressing transgenic lines, the increase may be related to the high turnover of OA, which ultimately leads to an increase in these elements.

Sclerotinia sclerotiorum is one of the most devastating, broad-based, necrotrophic, soilborne plant pathogens, which causes stem rot or white mould, with a host range of more than 400 species of plants, comprising crops, ornamental plants, trees and several weeds (Malenčič et al., 2010). Studies with OA-deficient mutants have shown that this molecule is a key suppressor of plant defences, thus allowing the fungus to evade recognition (Williams et al., 2011). Therefore, the ability of S. sclerotiorum to infect FvOXDC-expressing low OA-containing transgenic soya bean and grass pea was tested. Infection assay performed with detached leaf revealed a noteworthy delay in lesion development and more tolerance to S. sclerotiorum infection by the FvOXDC-expressing transgenic lines compared to the wild type (Figure 5). These results also demonstrated that the generation of fungal-tolerant soya bean and grass pea lines is also achievable by expressing FvOXDC. Previously, various transgenic plants, which can metabolize and/or degrade OA in an enzymatic manner, have been generated and shown to have enhanced tolerance to S. sclerotiorum (Cunha et al., 2010; Donaldson et al., 2001; Hu et al., 2003; Kesarwani et al., 2000; Walz et al., 2008). OA treatment suggested that FvOXDC is active in vivo as transgenic leaves of both soya bean and grass pea could resist the formation of brown lesion and yellowing caused by OA (Figure 1l).

Previous reports suggested that OA suppresses host defence by manipulating redox status (Cessna et al., 2000; Lehner et al., 2008; Williams et al., 2011). At the initial stage of disease establishment, OA induces a reducing environment in the host cells that suppresses host defence-related oxidative burst and...
callose deposition. However, at the later stage, OA promotes the generation of ROS in host tissue leading to programmed cell death which directly benefits the necrotrophic pathogens (Williams et al., 2011). On the contrary, non-pathogenic OA-deficient mutant is unable to alter host redox status (Cessna et al., 2000; Williams et al., 2011). Moreover, the effect of OA on host defence response might be mediated through phytohormone signalling pathways such as jasmonic acid (JA), ethylene (ET), abscisic acid (ABA) and salicylic acid (SA). It was suggested that OA induces JA, ET and ABA signalling pathways; however, it suppresses SA pathway in Brassica napus (Liang et al., 2009). Similarly, overexpression of OA-responsive WRKY family transcription factors revealed that JA/ET pathway is mainly active to defend Arabidopsis against S. sclerotiorum infection and OA stress (Chen et al., 2013). These observations are in agreement with an important role of the host JA/ET signalling pathway in response to necrotrophic fungal pathogens (Liang et al., 2008; Van Kan, 2006; Yang et al., 2007; Zhao et al., 2007). Although we have tested S. sclerotiorum tolerance of the FvOXDC-expressing transgenic lines of soya bean and grass pea (Figure 5), these transgenic lines might also exhibit an altered tolerance to other necrotrophic fungal pathogens such as Alternaria alternata, Botrytis cinerea. Interestingly, manipulating host OA level and we found no remarkable expression difference in the identified proteins (Figure S3a,b and Tables S1 and S2). To determine how a transgene might affect global gene expression or metabolism of the host plant, evaluation of transgenic plants can be performed at the levels of transcriptome, proteomics and metabolome (Ricroch et al., 2011). Previously, transcriptome profiling of about 24000 genes of transgenic Arabidopsis expressing nptIIIuidA revealed no noticeable difference with the wild type (El Ouakfaoui and Miki, 2005). Similarly, many researchers performed profiling of transcripts, proteins and metabolites of transgenic plants without any significant differences (Barros et al., 2010; Cheng et al., 2008; Coll et al., 2009; Kogel et al., 2010). In conclusion, we have developed genetically modified soya bean and grass pea with improved nutritional quality and enhanced tolerance to fungal pathogen without any adverse effect on plant growth and development and seed protein quality.

Experimental procedures

Construction of FvOXDC expression vectors

Overexpression of FvOXDC in soya bean and grass pea was performed using pSOVA (for soya bean and grass pea) and pSPM9 (for soya bean) constructs. To overexpress FvOXDC specifically in soya bean seeds, the coding sequence of FvOXDC was cloned under the regulation of the promoter of a soya bean gene encoding LEA protein GmPM9 by replacing the CaMV35S promoter of pSOVA (Kesarwani et al., 2000) with the GmPM9 promoter (Lee et al., 2000). pSPM9 was mobilized into Agrobacterium tumefaciens strain EHA105 using the helper strain HB101:: pRK2013 via the triparental mating technique (Van Haute et al., 1983).

Transformation and selection of transgenic events of soya bean

Cotyledonary node explants were excised from 7- to 10-day-old seedlings germinated on germination medium [Gamborg's B-5 basal medium (G5893) with 3% sucrose and 1 mg/L benzyl amino purine (BAP), pH adjusted to 5.6–5.8]. After agro-infection, cotyledonary node explants were transferred to co-cultivation medium [Gamborg's B-5 basal medium (G5893) with 3% sucrose, 200 μM acetosyringone, 0.25 mg/L gibberellic acid (GA3), 1 mM dithiothreitol (DTT), 1 mM sodium thiosulphate (STS), pH adjusted to 5.6) and maintained for 5 days. Further, explants were transferred to shoot induction and selection medium [Gamborg's B-5 basal medium (G5893), 3% sucrose] supplemented with 1.5 mg/L BAP, 0.25 mg/L GA3, 250 mg/L cefotaxime and 75 mg/L kanamycin. Three- to 7-cm long shoot buds were transferred to rooting media consisting of ½ MS basal media with 3% sucrose, 0.5 mg/L IAA, 50 mg/L kanamycin and 250 mg/L cefotaxime, pH 5.8. Plantlets with well-developed roots were taken out from the medium, and the roots were rinsed in water to remove agar and transferred to small pots containing sterilized agro peat and vermiculite (2 : 1).

Transformation and selection of transgenic events of grass pea

Embryo explants were used to transform FvOXDC gene to grass pea. Explants were excised from overnight-soaked seeds and then agro-infected and transferred to co-cultivation medium (Gamborg's B-5 basal medium, 3% sucrose, 0.5 mg/L NAA, 0.5 mg/L BAP, 1 mM DTT, 1 mM sodium thiosulphate and 0.2 mM acetosyringone, pH 5.6) and incubated at 23 °C for 5 days.
Further, explants were transferred to shoot induction medium (Gamborg’s B-5 basal medium, 3% sucrose with 0.5 mg/L NAA and 1 mg/L BAP, pH 5.6–5.8) and selected on 100 mg/L kanamycin. Subculturing was performed at an interval of 15 days on shoot induction medium till shoots appeared. Small plantlets were excised and transferred to root induction medium (Gamborg’s B-5 basal medium with 3% sucrose, 0.1 mg/L IBA, pH 5.6) for rooting. Transformed plants were maintained in the growth room before being transferred to the experimental plot.

Nucleic acid isolation and analysis
To confirm the integration of FvOXDC in the putative transgenic plant genome, genomic DNA was extracted from leaf tissue (100 mg) using the DNeasy plant mini kit (Qiagen, Duesseldorf, Germany). The stable integration of the FvOXDC gene in the transgenic soya bean and grass pea plants was determined via PCR using the forward and reverse primers (5’-GCGAAGTGCC-CATTGACCTCCAGC-3’) and (5’-CAGCTCTCTCIAAGCAC-CAGGCTC-3’), respectively.

Immunodetection of FvOXDC
Crude proteins were extracted from soya bean and grass pea transgenic plants and their respective controls. From each sample, 100 μg of total protein was then separated on a 12.5% SDS–polyacrylamide gel electrophoresis. Proteins from SDS–PAGE gel were transferred onto nitrocellulose Hybond membrane by electrotransfer (GE Biosciences, Little Chalfont, UK). The membrane was probed with affinity-purified oxalate decarboxylase polyclonal antibody in combination with alkaline phosphatase-conjugated goat anti-rabbit IgG, using NBT-BCIP reaction.

Decarboxylation assay
The decarboxylation activity of FvOXDC was determined using 14C-oxalic acid (GE Biosciences) as a substrate using a method described previously (Mehta and Datta, 1991). 100 μg of soluble protein from each biological replicate was used for the decarboxylation assay. Samples were analysed in triplicate, and the mean value was calculated. One unit of decarboxylase activity is equivalent to 1 μmol/m L 14C-CO2 produced at 37 °C. The activity data were expressed as the means ± SD of at least three independent experiments.

OA treatment to leaves
The assay was performed according to Kesarwani et al. (2000) with slight modifications. Young leaves (three each) were detached from transgenic (soya bean/grass pea) and control (soya bean/grass pea) plants. Both transgenic and control leaves were then immediately dipped in 20 mM OA solution pH 4.0. Negative control was imposed by dipping the leaves in water. All the leaves were incubated in a plant growth chamber at 22–24 °C under 16-h light and 8-h dark photoperiod for 24 h.

Extraction and determination of OA and formic acid
Oxalic acid and formic acid were quantified using OA and formic acid detection kit according to the manufacturer’s protocol (Boehringer Mannheim/R-Biopharm, Darmstadt, Germany). Briefly, each sample was crushed into fine powder using mortar and pestle and then further suspended in 500 μL of MQ water. Homogenization was performed using a PRO 200 homogenizer for five cycles of 30 s each. Homogenate was then centrifuged at 13 000 g for 15 min at room temperature, and the supernatant was collected. Oxalate and formate contents were measured using 100 μL of the supernatant in triplicate, and the absorbance was read at 590 and 340 nm, respectively.

Determination of β-ODAP
Estimation of β-ODAP content from the transgenic and control plants was performed according to Rao (1978). To 100 μL of seed extract, 200 μL of 3 N KOH was added and the tubes were placed on boiling water bath for 30 min. After alkali hydrolysis, 700 μL of water was added to the tubes so that the final volume of the reaction is 1 mL followed by the addition of 2 mL o-phthalaldehyde (OPT) reagent. Samples were mixed and allowed to stand for 15 min to develop yellow colour. The absorbance was measured at 420 nm.

Determination of calcium and other micronutrients
Total calcium and other micronutrients such as magnesium, zinc, iron and manganese were analysed by energy-dispersive X-ray fluorescence analysis (EDXRF; PANalytical Epsilon 5, Almelo, The Netherlands). EDXRF gives unique spectra for each sample analysed. Seeds were grounded into fine powder and dried; then, 60 mg of dried seed powder was used to analyse the total calcium, magnesium, zinc, iron and manganese. A specific X-ray fluorescence signal emitted by the atoms after the photoelectric ionization was measured, and the radiation intensity of each element signal, which is proportional to the concentration of the element in the sample, is recalculated internally from a stored set of calibration curves and can be shown directly in concentration units.

Morphological characterization and photosynthetic activity
Plants were grown in the field, and the experiment was arranged in a randomized block design with three replicates. Plants were regularly watched for their morphological characters such as plant height, size of leaves, number of pods per plant and number of seeds per pod. Photosynthetic rates were measured with a GFS3000 portable photosynthesis measurement system (Walz, Effeltrich, Germany) after 60 days since they were planted. The experiment was performed at standard atmospheric (360 ppm CO2), light conditions (750 μmol/m²/s), and humidity was maintained at 70%. Photosynthetic rate of the leaf was measured on the basis of single-leaf measurement experiment which was conducted on three different leaves in each plant.

Detached leaf assays
To analyse the S. sclerotiorum infection in transgenic with respect to wild type, detached leaf assay was performed according to Cunha et al. (2010) with minor modifications. S. sclerotiorum were grown in potato dextrose broth (PDB) at 22 °C with 150 rpm till the growth of mycelial ball. Then, 2 g of mycelial ball was homogenized into 4 mL of 1% PDB and 10 μL of mycelial suspension was inoculated on detached leaves of transgenic soya bean and grass pea as well as to their wild-type counterparts. Leaves were photographed at three different times (24, 72 and 110 h), and images were used to measure the infected area using the NIS-Element AR 3.2 64-bit software of multiobjective stereo zoom (AZ-100) microscope (Nikon Instruments Inc., Melville, NY, USA).

Protein isolation, trypsin digestion, iTRAQ 4-plex labelling and data analysis
Total proteins were isolated from soya bean and grass pea seeds using Plant Total Protein Extraction Kit (Sigma-Aldrich, St Louis,
MO, USA, Catalog number PE0230) according to the manufacturer’s instructions. For proteomic analysis, biological duplicates from each of the two conditions (wild type and transgenic line) studied were handled simultaneously for iTRAQ labeling (AB Sciex, Foster City, CA) as per the manufacturer’s instruction. Briefly, 70 μg of protein from each sample was used for modified trypsin (Promega, Madison, WI, USA, V511) and incubated for 16 h at 37 °C in a 1 : 10 trypsin-to-protein ratio (Maity et al., 2014). To label tryptic-digested peptides of each sample, different iTRAQ tags such as 114, 115, 116 and 117 were used following the manufacturer’s protocol (AB Sciex). The data were assimilated in an information-dependent acquisition (IDA) mode Analyst TF 1.6 software (AB Sciex) using the triple ToF 5600 (AB Sciex). For the identification of proteins, Paragon algorithm in a ‘Thorough ID’ search mode was employed against the SGD-Saccharomyces cerevisiae reference data set (6750 protein sequences). The search parameters allowed modifications by IAA at cysteine residues, 4 plex peptide iTRAQ labelling of the N-termini of peptides and of the side chains of lysine. We applied 1% global protein-level FDR for protein identification.

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References


Reduced oxalic acid in fungal tolerant legumes


**Supporting information**

Additional Supporting information may be found in the online version of this article:

**Figure S1** Transformation and regeneration of soya bean cotyledonary nodes under kanamycin selection, and recovery of transgenic plant.

**Figure S2** Transformation and regeneration of grass pea embryos as explants under kanamycin selection, and recovery of transgenic plant.

**Figure S3** Protein profiling of soya bean and grass pea seeds with iTRAQ technique.

**Table S1** List of soya bean proteins identified and quantified through iTRAQ.

**Table S2** List of grass pea proteins identified and quantified through iTRAQ.