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PRpnp, a novel dual activity PNP family protein improves plant vigour and confers multiple stress tolerance in *Citrus aurantifolia*

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Summary

Under field conditions, plants are often simultaneously exposed to several abiotic and biotic stresses resulting in significant reductions in growth and yield; thus, developing a multi-stress tolerant variety is imperative. Previously, we reported the neofunctionalization of a novel PNP family protein, *Putranjiva roxburghii* purine nucleoside phosphorylase (PRpnp) to trypsin inhibitor to cater to the needs of plant defence. However, to date, no study has revealed the potential role and mechanism of either member of this protein group in plant defence. Here, we overexpressed *PRpnp* in *Citrus aurantifolia* which showed nuclear-cytoplasmic localization, where it functions in maintaining the intracellular purine reservoir. Overexpression of *PRpnp* significantly enhanced tolerance to salt, oxidative stress, alkaline pH, drought and two pests, *Papilio demoleus* and *Scirtothrips citri* in transgenic plants. Global gene expression studies revealed that *PRpnp* overexpression up-regulated differentially expressed genes (DEGs) related to ABA- and JA-biosynthesis and signalling, plant defence, growth and development. LC–MS/MS analysis validated higher endogenous ABA and JA accumulation in transgenic plants. Taken together, our results suggest that *PRpnp* functions by enhancing the endogenous ABA and JA, which interact synergistically and it also inhibits trypsin proteases in the insect gut. Also, like other purine salvage genes, *PRpnp* also regulates CK metabolism and increases the levels of CK-free bases in transgenic Mexican lime. We also suggest that *PRpnp* can be used as a potential candidate to develop new varieties with improved plant vigour and enhanced multiple stress resistance.

Introduction

Being sessile organisms, plants are constantly confronted by several abiotic and biotic stresses. Over the course of evolution, plants have developed complex defence machinery that produces numerous defensive proteins in response to adverse conditions. One such group of proteins comprises wound-inducible (WI) and vegetative storage proteins (VSPs). VSPs are found in all plant species and are primarily known to serve as nitrogen reserves in vegetative tissues. VSPs also possess sequence homology with pathogenesis-related proteins such as abscisic acid (ABA) and jasmonic acid (JA) responsive proteins (Tegeder and Masclaux-Daubresse, 2018). The defensive role of several WI proteins and VSPs have been reported, such as a sugarcane WI protein '*SUGERWIN2*' showed antipathogenic properties (Medeiros *et al*., 2012), JA-induced *Arabidopsis* VSP, *AtVSP2* exerts insecticidal activity against coleopteran and dipteran insects (Liu *et al*., 2005), and recently, a VSP, maize mesophyll lipoxygenase *ZmLOX6*, improved plant drought tolerance upon its overexpression (Abbaraju *et al*., 2022). However, the defensive role of several other similar proteins remained largely unknown (Verma *et al*., 2017).

Our research group was the first to identify and characterize a ~34 KDa *Putranjiva roxburghii* purine nucleoside phosphorylase (PRpnp) protein, isolated from the seeds of *P. roxburghii*, a medicinal plant belonging to the *Euphorbiaceae* family. Initially, PRpnp was reported as a *P. roxburghii* trypsin inhibitor (PRTI) due to its nature as a competitive inhibitor of trypsin (Chaudhary *et al*., 2008). Later, we performed a detailed sequence analysis of *PRpnp* and reported the existence of a group of similar proteins that displayed sequence homology and evolutionary linkage to stress-induced proteins characterized as WI protein, VSPs and bark storage proteins. All the group members contain a purine nucleoside phosphorylase–uridine phosphorylase (PNP–UDP) family domain, disrupted with a stretch of amino acids. PRpnp showed an insertion of 46-amino acid residues in its PNP–UDP domain, which is responsible for its trypsin inhibitory nature and, thus, it possesses dual activity, that is, PNP and protease (trypsin) inhibitory activity. The insertion of a trypsin inhibitory loop is the case of neofunctionalization of PRpnp to a potent trypsin inhibitor (TI) to cater to the needs of plant defence (Verma *et al*., 2017, 2022).

Purine nucleoside phosphorylase (PNP; E.C.-2.4.2.1), a salvage pathway enzyme of purine biosynthesis that catalyses a reversible reaction to produce free purine base and (2′-deoxy) ribose-1-phosphate (Verma *et al*., 2017). Purine nucleotides are the building blocks of nucleic acids, energy carriers and cell signalling molecules and also play fundamental roles in metabolism (Zhao

et al., 2015a). Purine salvage enzymes, including PNPs, are also involved in cytokinin (CK) metabolism (Ashihara *et al*., 2018). Stress conditions such as wounding require an abundant supply of adenine nucleotides; thus, the level of purine nucleotides and relative expression intensity of purine salvage genes and their *in vitro* activities over *de novo* were increased in wounded potato tuber slices (Katahira and Ashihara, 2006).

Protease inhibitors (PIs) are natural plant defence proteins that form stable protease-inhibitor complexes with their target proteases, thereby inhibiting them (Srinivasan *et al*., 2009). The defensive role of several PIs, including TIs, in response to both abiotic and biotic stresses, is widely known (Srinivasan and Kirti, 2012). Numerous transgenic plants overexpressing different PIs have been produced with enhanced stress tolerance (Clemente *et al*., 2019; Srinivasan and Kirti, 2012). The recurrent stress challenges in plants have pushed them to develop an efficient and resilient defence system. To accomplish this, numerous structural and functional changes in plant proteins have been observed during the course of evolution (Bartlett and Whipple, 2013). Similarly, PRpnp and other homologous proteins evolved and attained a disruption in the PNP–UDP domain (Verma *et al*., 2017, 2022). The purine salvaging capacity and neofunctionalization of PRpnp to TI, along with its evolutionary linkage to other plant defence proteins, prompted us to explore its defensive role against multiple environmental stresses.

Citrus is one of the major fruit crops, grown in the tropical and subtropical regions of more than 140 countries and includes many species of economic importance, such as limes, oranges, lemons, grapefruit and tangerines (Liu *et al*., 2012). Among the three classes of limes, *Citrus aurantifolia* is the globally predominant natural hybrid, with India, China and Mexico as its prime producers; however, its cultivation is remarkably limited by numerous abiotic and biotic stresses (Donkersley *et al*., 2018). Under field conditions, *C. aurantifolia* experiences several stresses simultaneously, which hampers its growth and decreases the economic yield; therefore, developing a multi-stress tolerant variety is imperative. In this study, PRpnp showed nuclear-cytoplasmic localization and was constitutively overexpressed in Mexican lime. Transgenic plants showed enhanced tolerance to salt, oxidative stress, alkaline pH, drought and two pests, *Papilio demoleus* and *Scirtothrips citri*. Furthermore, we found that *PRpnp* functions by enhancing the endogenous ABA and JA, which interact synergistically and it also inhibits trypsin proteases in the insect gut. Other important functions of *PRpnp* include the maintenance of intracellular purine reservoir and CK homeostasis.

Results

PRpnp localizes to the nucleus and cytosol

The apparent molecular weight (MW) of PRpnp protein is ~34 kDa and the pI value corresponds to 5.44. The modular structure of PRpnp protein is illustrated (Figure 1a). The subcellular localization and functionality of a protein are likely interconnected (Yu *et al*., 2006). To examine the subcellular localization of PRpnp, we transiently expressed PRpnp-GFP fusion protein in *Nicotiana benthamiana* leaves. Upon 2 days post infiltration (dpi), laser-scanning confocal microscopy detected the green fluorescent signal of PRpnp-GFP protein in both the nucleus and cytosol (Figure 1b: IV). The RFP was used as a nucleocytoplasmic marker (Figure 1b: II) and the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Figure 1b: III). The experiment was repeated thrice and showed the same subcellular localization for PRpnp (Figure 1b).

Generation and confirmation of transgenic *C. aurantifolia* Cv. Pramalini

To end the speculation regarding its defensive role, *PRpnp* was chosen as an overexpression target and the *Agrobacterium*-mediated transformation method was used to transform *C. aurantifolia* Cv. Pramalini, First, we successfully assembled the expression cassette in pBSSK(+) (Figure S1a) and subsequently subcloned it to the binary vector pCAMBIA2301 (Figure S1b,c). Putative transgenic shoots with kanamycin resistance and positive GUS histochemical activity were obtained. We observed no GUS activity in control. Finally, to recover the whole transgenic plant, the putative transgenic shoots were either *in vitro* micrografted or cultured on rooting media. The stages of transgenic Mexican lime development are depicted (Figure S2).

To select the plants for subsequent experiments, we characterized the putative transgenic plants by duplex PCR (Figure S3a) and quantitative real-time PCR (qRT-PCR) (Figure S3b), which showed successful *PRpnp* gene integration and expression, respectively. Further, we detected bands corresponding to the expected MW of PRpnp protein, that is, ~34 kDa, which marked its expression in transgenic plants by Western blot analysis. No equivalent band was detected in WT (Figure S4a). Based on the results of qRT-PCR and Western blot analysis, transgenic plants that showed decent *PRpnp* expression and protein accumulation were chosen for further experiments in this study. All the GUS-positive transgenic Mexican limes that were developed in this study and their recovery method are documented (Table S1).

The total leaf protein fractions (200 μg) from WT and transgenic plants were isolated to assess PNP and trypsin inhibitory activity. To confirm the presence of PNP activity, we subjected the protein isolates to react with inosine and measured OD at 293 nm. We recorded a comparative increase in absorptions in the reaction mixture of transgenic plants A2, A6 and A11, which were, respectively, 3.6-, 1.95- and 1.64 times higher than WT (Figure S4b: I). Trypsin inhibitory activity was measured as percentage inhibition of trypsin (I%). Previous report quantifying I % from the protein fraction of transgenic plants is also available (Senthilkumar *et al*., 2010). Transgenic plants A2, A6 and A11, showed 68.41%, 56.54% and 48.41% inhibition percentages, respectively (Figure S4b: II). The presence of both PNP and trypsin inhibitory activity confirms that PRpnp functions appropriately in transgenic *C. aurantifolia*.

Leaf disc senescence assay of transgenic *C. aurantifolia* shows tolerance to multiple abiotic stresses

Six transgenic plants A2, A6, A11, A28, A30 and A32, with variable *PRpnp* expression levels, were selected for leaf disc senescence assay. Leaf discs from healthy and fully expanded leaves of WT and transgenic plants were exposed to different stress solutions (200 and 300 mM NaCl for salt stress; 48 and 72 h, 50 mM H_2O_2 for oxidative stress; 48 h, and pH = 9 for alkaline stress; 120 h) and was compared to control. The leaf discs of WT showed partial or complete bleaching depending upon the type/duration of stress applied; however, under the same conditions, no or significantly less bleaching was noticed in transgenic leaf discs. All the leaf discs remained green under control conditions (Figure 2a–c). *PRpnp* overexpressing transgenic plants showed a clear advantage in overcoming the deleterious effect of NaCl, H_2O_2 and high pH. Further, biochemical

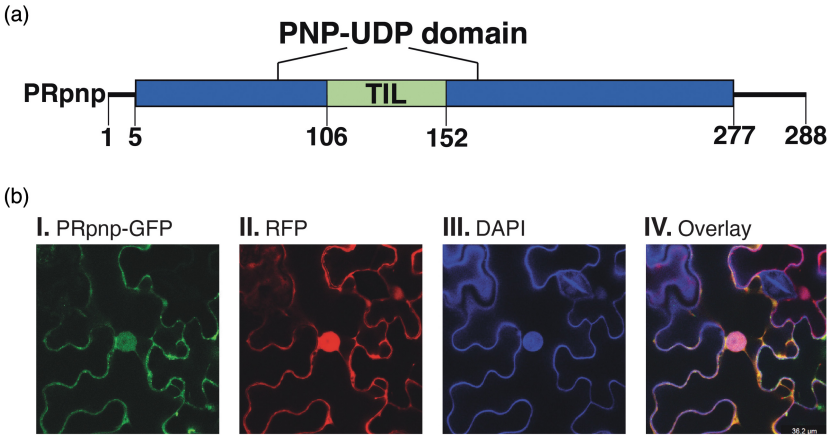

Figure 1 Modular structure and subcellular localization of PRpnp. (a) PRpnp belongs to the PNP family and consists of 288 amino acids. The PNP–UDP family domain in PRpnp is disrupted by a 46 amino acid insert. The attainment of trypsin inhibitory property through insert does not disturb the tertiary structure of PRpnp, suggesting its neofunctionalization due to the evolutionary needs of plant defence. TIL-Trypsin inhibitory loop. (b) PRpnp was localized in the nucleus and cytosol. *Agrobacterium* strain GV3101 harbouring pB7FWG2-35 S::PRpnp-GFP plasmid was infiltrated in *N. benthamiana* pavement cells. Live cell images were captured with laser-scanning confocal microscopy. (I) Transient expression of PRpnp-GFP protein, (II) RFP fluorescent signal, (III) staining of the nuclei by DAPI and (IV) merged image of PRpnp-GFP, RFP and DAPI channel. The GFP, RFP and DAPI channels were excited at 488, 552 and 405 nm. The scale bar represents 36.2 μm.

investigations to evaluate the degree of stress-triggered cellular damage, the chlorophyll (a and b) and carotenoid were estimated. After imposing the stresses, we noticed significantly higher pigment retention in all the transgenic plants over WT. Furthermore, among all the transgenic, plant A2 showed the highest, whereas A28 showed the lowest retention of tested pigments under all the stress conditions (Figure 2d–f). Under salt stress (300 mM; 72 h), oxidative stress and alkaline stress, transgenic plants A2 could retain 2.3-, 3.3- and 1.99 times; 1.99-, 2.24- and 1.70 times; 2.48-, 2.79- and 2.08 times while plant A28 showed 1.48-, 2.25- and 1.39 times; 1.35-, 1.25- and 1.11 times; 1.29-, 1.38- and 1.22 times higher Chlorophyll a (chl a), Chlorophyll b (chl b) and carotenoids content, respectively over WT (Figure 2d–f). Overall, these results documented the usefulness of *PRpnp* overexpression in providing multiple stress resistance in transgenic plants.

Overexpression of *PRpnp* improves tolerance to high salt concentration also at the whole plant level

Generally, leaf disc senescence assay involves using a higher concentration of NaCl to see the results in a shorter period. Thus, it does not reflect the actual tolerance limits of transgenic plants under natural conditions (Bhaskaran and Savithramma, 2011). Therefore, to further validate the stress-responsive role of *PRpnp* at the whole plant level, we treated WT and selected transgenic *C. aurantifolia* A17, A19 and A21 with 75 mM NaCl for 45 days. Since we harvested leaves from WT and transgenic plants for leaf disc senescence and other detached leaf assays (described in later sections), and due to the long juvenile period in *C. aurantifolia*, we used different sets of plants to assess salt and drought tolerance at whole plant level. However, the expression of *PRpnp* transcript (Figure S3b) and protein (Figure S4a) in every plant was determined before proceeding. The plants selected for assessing salt and drought stress tolerance under soil conditions were recovered by rooting. Plants of similar age and height were selected for this study. Also, all the selected transgenic plants were clonally propagated to generate at least 4–6 identical plants using the standard method of citrus node culture to perform three biologically independent experiments. From the third week of treatment, WT started showing severe salt stress-induced damage including leaf wilting that was simultaneously accompanied by leaf rolling, tip-initiated leaf burning and leaf abscission which subsequently caused plant death. By contrast, no severe symptoms were detected in transgenic plants (Figure 3a). Moreover, the physiological parameters including proline and malondialdehyde (MDA), were estimated in WT and transgenic plants at 0 days, 25th day, 40th day and 45th day. Being a compatible osmolyte and reactive oxygen species (ROS) scavenger, increased proline levels have been widely proven to play a crucial role in salt and drought tolerance (Kavi kishor and Sreenivasulu, 2014). The strength of plant responses to adverse environmental conditions is marked by MDA, an indicator of lipid peroxidation of cell membranes (Song *et al.*, 2022). Thus, increased MDA levels could be determinantal to plants. The same-day comparison showed that the significant increase in proline was higher, and MDA was lower in transgenic plants relative to WT plants. No significant differences were observed at day 0. Proline accumulation in *PRpnp* overexpressing plants A17, A19 and A21 showed 136.68, 112.97 and 82.30% increase (%) (Figure 3b) while percent decrease (%) in MDA levels were 57.76, 41.84 and 43.47 (Figure 3c) relative to WT, at 45th day of salt stress treatment. With no significant sign of salt-elicited leaf damage, high proline content and low MDA levels, the results show that transgenic plants are tolerant toward salt stress compared to WT. Thus, the overexpression of *PRpnp* significantly alleviated the deleterious effects of salt stress in transgenic *C. aurantifolia*.

Overexpression of *PRpnp* improves tolerance to drought

To examine the role of *PRpnp* in regulating drought stress tolerance, watering to WT and transgenic citrus, A22, A24 and A25 were withheld for 25 days. All the plants used in the study were cultivated under a fully watered regime before stress treatment. No phenotypic differences were observed among the

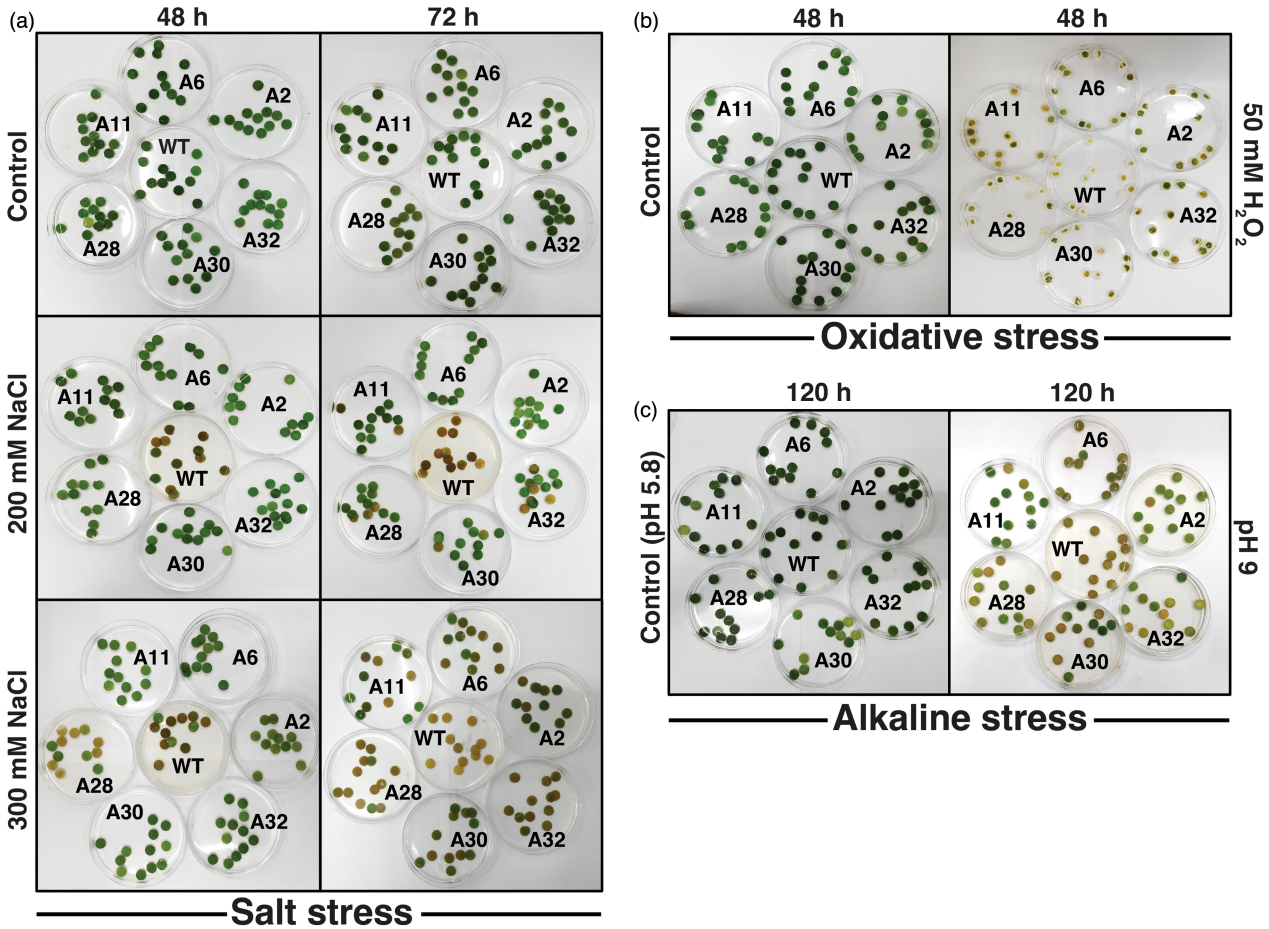

Figure 2 Leaf disc senescence assays showed enhanced multiple stress tolerance in *PRpnp* transgenic *Citrus aurantifolia*. (a) Leaf discs were floated in media supplemented with 200 mM NaCl and 300 mM NaCl for 48 and 72 h. (b) Leaf discs were evaluated against oxidative stress by adding 50 mM H_2O_2 to the media for 48 h. (c) Response of leaf discs was also tested against alkaline pH. To mimic higher alkalinity, the pH of the media was set to 9. For control, the leaf discs were incubated in ½ strength Hoagland solution; pH = 5.8. In all the tested conditions, WT leaf discs showed a distinctly high level of bleaching, thus, indicating their low-stress tolerance compared to transgenic. (d, e, f) Chlorophylls (a and b) and carotenoid content of WT and transgenic leaf discs were estimated and compared under control and stressed conditions; (d) salt stress, (e) oxidative stress and (f) alkaline stress. All the transgenic leaf discs showed more chlorophylls and carotenoid content under stressed conditions over WT. Data represent means ± SE (n = 3) of three biologically independent experiments. Significant differences between WT and transgenic lines were calculated using Student's t-test, *P-value <0.05 and **P-value <0.01.

plants in the first week of drought. However, by the second week, severe symptoms of water deficiency, such as light green and yellow-green foliage, leaf wilting, curling and drying followed by twig dieback, were observed in WT, while transgenic plants showed no significant drought-induced symptoms. New foliage development in A22 and A24 during drought indicated superior growth and metabolism rates over WT. Normal watering conditions were resumed after 25 days in all the plants for stress recovery, and observations were made after 15 days. We observed poor recovery in WT upon rewatering; however, transgenic plants rejuvenated quickly (Figure 4a). To combat the episodic drought or watering pulse, the plant usually abandons its older parts and produces new ones upon rewatering (Xu *et al.*, 2010). We observed a similar pattern of drought stress survival in *PRpnp* transgenic plants during recovery (Figure 4a). The phenotypes of WT plants which were in contrast to the performances of *PRpnp* overexpressing plants under the same stress conditions supported the positive role of *PRpnp* in response to drought. This was further validated by quantification of proline, MDA and relative water content (RWC). At the end of treatment, with 120.037%, 79.42% and 94.35% increase in proline accumulation (Figure 4b) and 56.06%, 40.17% and 50.20% decrease in MDA levels (Figure 4c), transgenic plants A22, A24 and A25, respectively, performed better in tolerating drought, compared to WT. Additionally, A22 maintained 50.83%, followed by 37.80% and 45.07% RWC in A24 and A25, while WT could only retain 22.25% (Figure 4d). Together, these results indicate that *PRpnp* overexpression enhances drought tolerance in transgenic *C. aurantifolia* relative to WT.

Overexpression of *PRpnp* confers resistance to *P. demoleus* larvae

TIs are well known to confer resistance to Lepidopteran in transgenic plants (Srinivasan *et al.*, 2009; Srinivasan and Kirti, 2012). Since PRpnp also possesses protease inhibitory activity, we hypothesized that it could promote pest resistance. To investigate this, leaves of three transgenic plants, A2, A6 and A11, were challenged to five-second instar larvae of

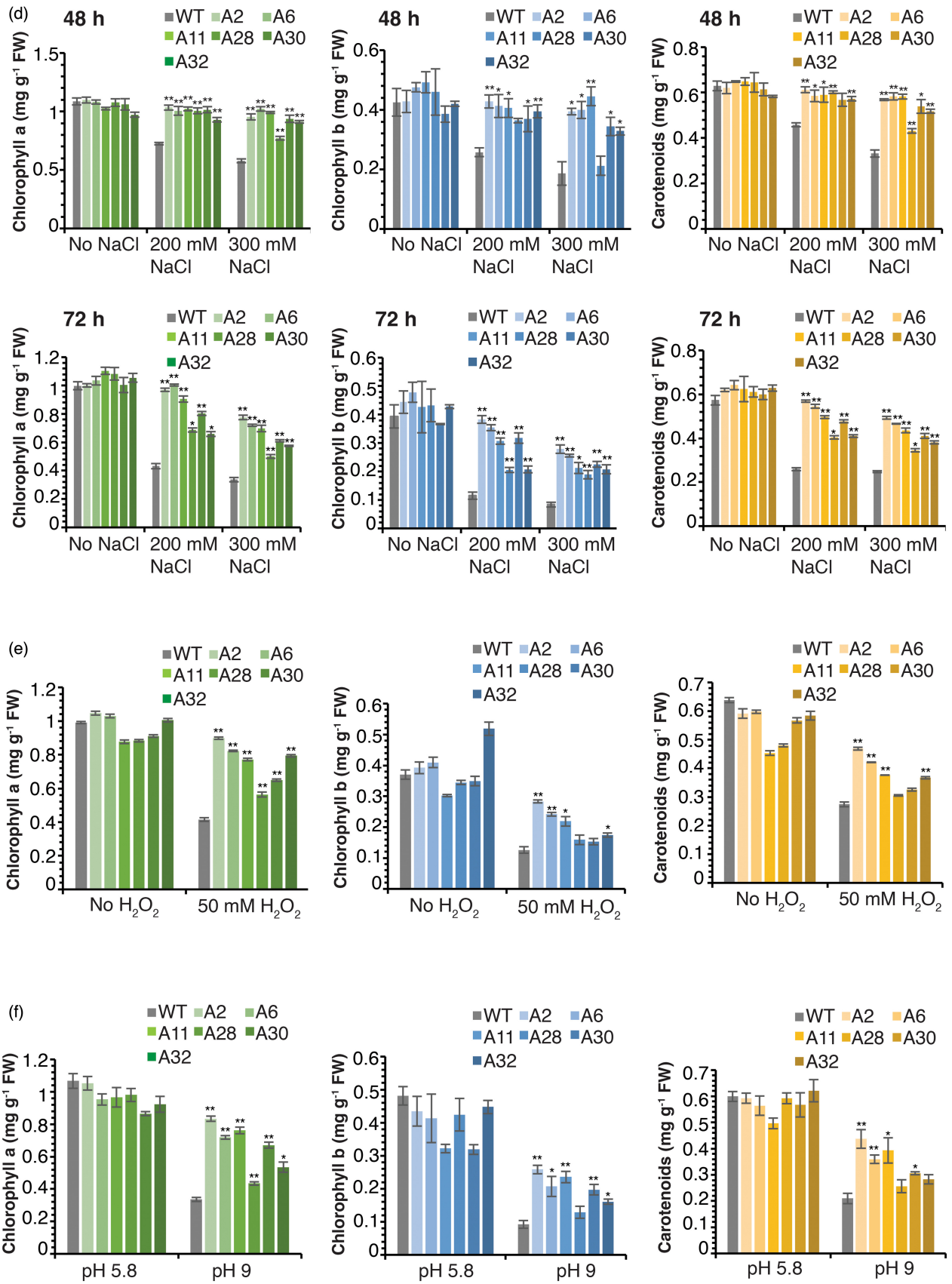

Figure 2 (continued)

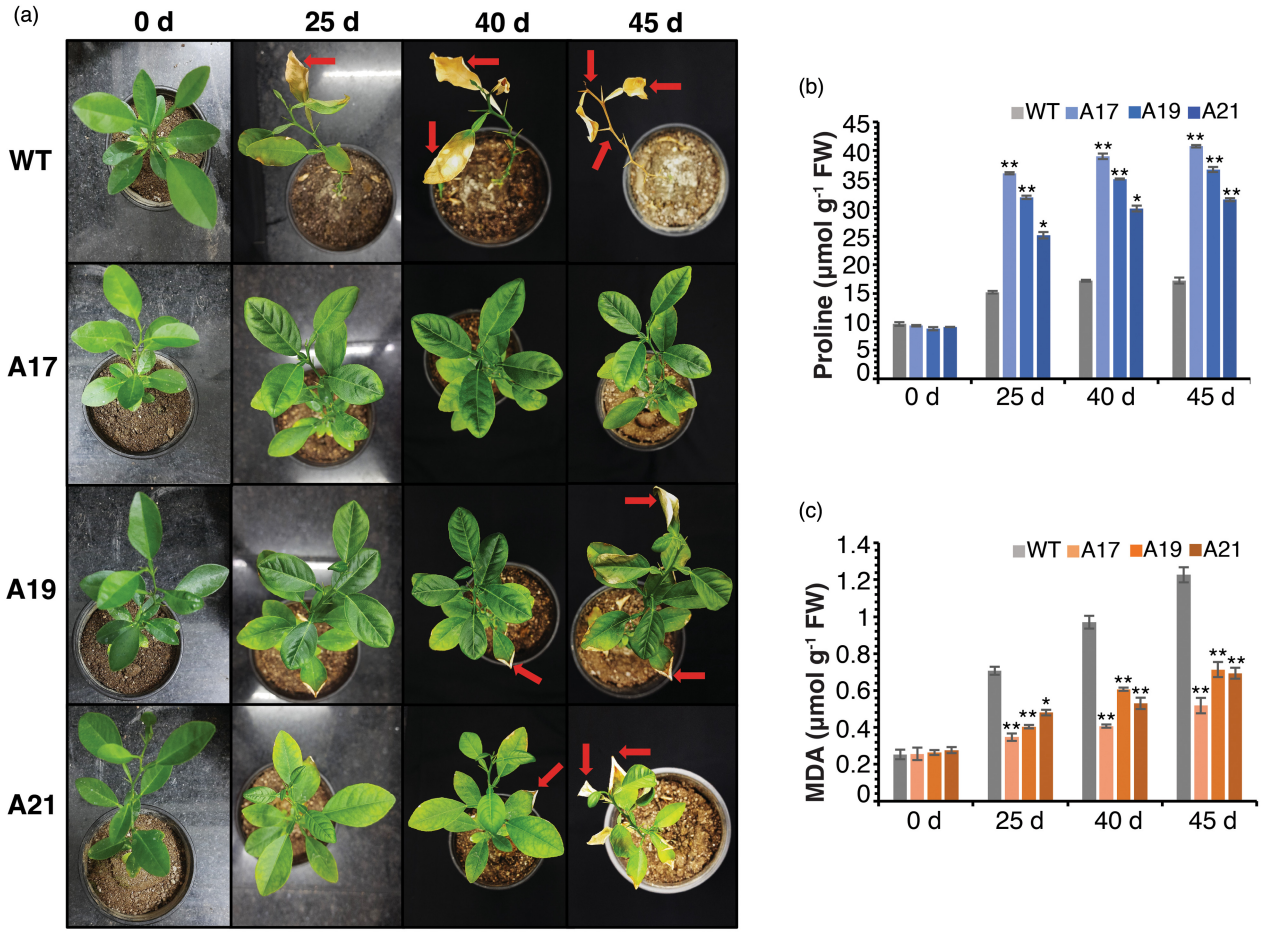

Figure 3 *PRpnp* overexpression enhances salt tolerance also under soil conditions. (a) Photographs of growth of WT and transgenic plant under salt stress at indicated time points. 0 day (0 d) represents plant conditions before stress treatment. Severe salt stress-induced symptoms in WT ultimately led to plant death; however, transgenic plants survived with no or little effect. Red arrows indicate salt stress-induced damage. (b) proline and (c) malondialdehyde (MDA) content in WT and A17, A19 and A21 transgenic plants at regular intervals. Transgenic plants showed significantly higher levels of proline and lower levels of MDA relative to WT. Data represent means ± SE ($n = 3$) of three biologically independent experiments. Significant differences between WT and transgenic lines were calculated using Student's *t*-test, **P*-value <0.05 and ***P*-value <0.01.

P. demoleus and compared with the control. The visible comparative growth of WT-fed larvae was superior to transgenic ones (Figure 5a–c). This was supported by the declined average body weights of A2, A6 and A11 fed larvae which were, respectively, 5.47-, 4.48- and 3.25 times less than control at 12 day-post-hatching (12 DPH). Comparatively, transgenic-fed larvae gained significantly less body weight between 9 and 12 DPH; however, WT-fed larvae displayed normal growth rates (Figure 5b–d). The declined body weights were directly related to reduced herbivory. At 12 DPH, 53% larval mortality was recorded in A2, followed by A6 and A11 fed larvae which were, respectively, 46% and 26%, while no larval mortality was observed in the WT plant (Figure 5e). From the results, it was apparent that overexpression of the *PRpnp* gene in *C. aurantifolia* plants improves resistance to *P. demoleus* larvae.

Overexpression of *PRpnp* promotes resistance to adult *S. citri*

We further extended our study by investigating the insecticidal properties of *PRpnp* against another pest, adult *S. citri*. To evaluate this, a detached leaf bioassay of WT and transgenic plants, A2, A6 and A11, was performed. Each leaf was infested with 18–20 thrips (Figure 6a,b). The surviving thrips were counted manually, and their mortality was recorded every 24 h for 3 days. Few WT- and transgenic-fed thrips were imaged under the microscope after 72 h (Figure 6c,d). The declining population of adult citrus thrips and increased corrected mortality (%); (CM%) were linked to the insecticidal effects of *PRpnp* overexpression in transgenic plants. The maximum recorded CM (%) after 72 h was 59.11, 53.17 and 40.52, respectively, for A2, A6 and A11 plants (Figure 6e). Taken together, the reduced survival rates of adult citrus thrips upon transgenic plants, compared to control, confirmed the advantage of *PRpnp* overexpression in *C. aurantifolia*.

De novo transcriptome profiling analysis identified highly enriched GO terms and DEGs in WT and *PRpnp* transgenic *C. aurantifolia* under control conditions

The multiple stress resistance phenotype of transgenic *C. aurantifolia* prompted us to investigate how overexpression of *PRpnp* affects the global gene expression profiles. To accomplish this, we

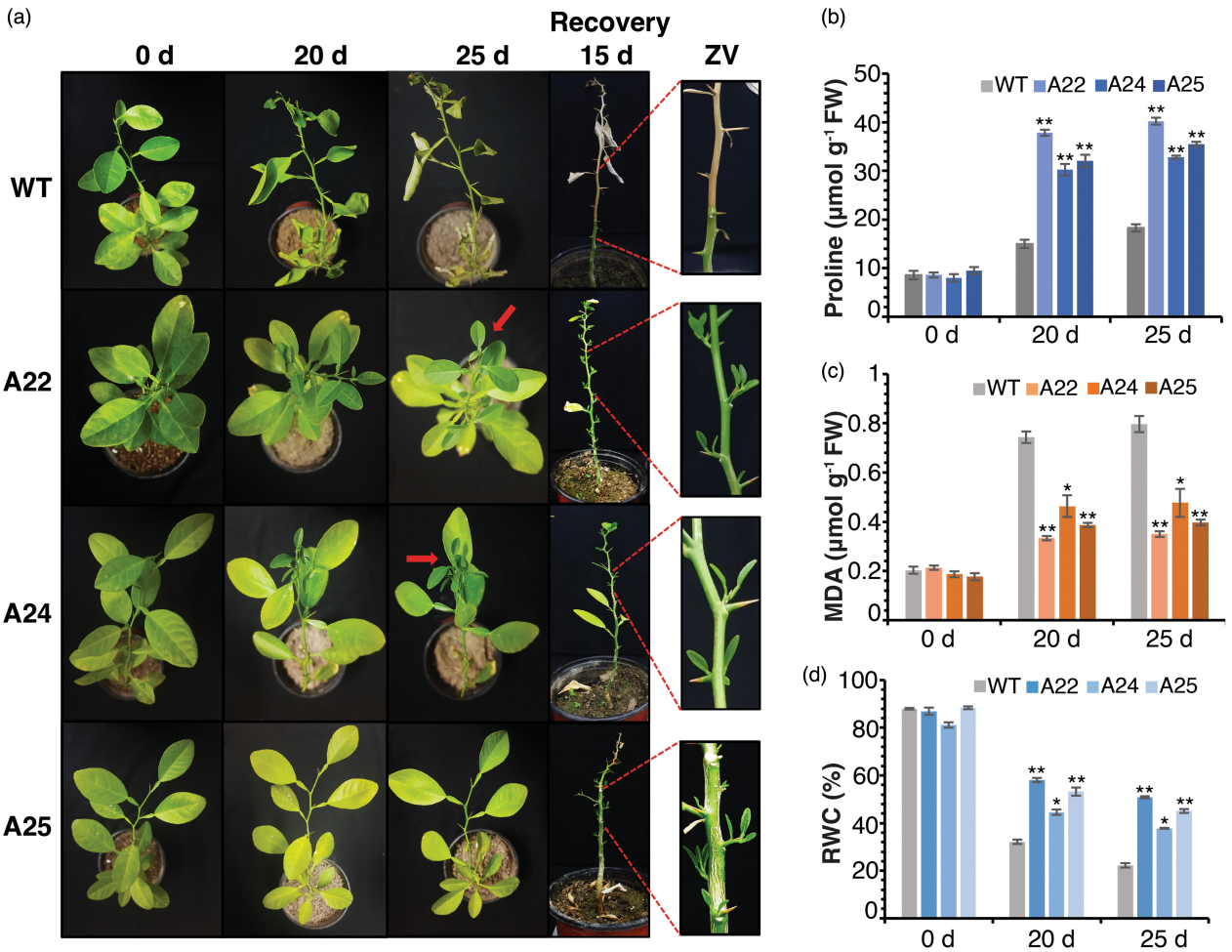

Figure 4 *PRpnp* overexpression enhances drought tolerance. (a) Phenotype of WT and transgenic plants under drought stress at the indicated time points. WT showed early and deleterious drought-induced symptoms along with delayed recovery upon reirrigation. However, transgenic plants survived all the phases of the experiment and showed quick recovery. Red arrows indicate new leaf development during drought stress. Zoomed view (ZV) shows delayed recovery in WT and new foliage development in transgenic plants upon rewatering. (b) proline content, (c) lipid peroxidation rates (MDA) and (d) relative water contents (RWC) of WT and A22, A24 and A25 transgenic plants at regular intervals. Transgenic plants showed relatively higher proline levels and RWC and a lower increase in MDA levels. Data represent means ± SE (n = 3) of three biologically independent experiments. Significant differences between WT and transgenic lines were calculated using Student's t-test, *P-value <0.05 and **P-value <0.01.

performed high-throughput RNA-seq experiments on WT and transgenic plants under control conditions. A total of ~52 million raw reads were generated, with approximately ~25 million and ~27 million high-quality reads for WT and transgenic plants, respectively. Out of 6348 identified differentially expressed genes (DEGs), 3308 DEGs were up-regulated, and 3040 DEGs were down-regulated in WT and transgenic samples (Table S2). The principal component analysis (PCA) plot analysis validated the discrimination between the transcriptome profiles and showed that the WT samples were clustered together, and there was a significant sample-to-sample distance between WT and transgenic counterparts (Figure S5a). Furthermore, we illustrated the distribution of up-regulated and down-regulated DEGs by volcano plot (Figure S5b).

We performed Gene Ontology (GO) enrichment analysis and functional annotation of DEGs by BiNGO in Cytoscape software (Figure S6). The network analysis showed that GO of 'response to hormone stimulus', response to abiotic stimulus', 'response to biotic stimulus', 'cellular nitrogen compound metabolic process', 'anatomical structure development', 'embryonic development', 'root morphogenesis', 'transport', 'potassium ion transport', 'calcium ion transport', 'cell cycle', 'plastid organization', 'chloroplast organization', and 'ion homeostasis' were significantly enriched in the up-regulated DEGs (Figure S6a–e) whereas GO terms belonging to 'anatomical structure morphogenesis', 'post-embryonic development', 'shoot morphogenesis', 'cell wall organization or biogenesis' and 'protein modification process' were enriched in the down-regulated DEGs (Figure S6f–h).

The variation in the expression of selected DEGs is shown in the heat maps, and their relative expression levels are depicted in different colors (Figure S7). DEGs analysis suggested that the genes belonging to phytohormone biosynthesis and signalling (*AAO3*, *PYL9*, *LOX6*, *OPR3*, *4CLLs*, *ADK2*, *LOG2*), plant defence

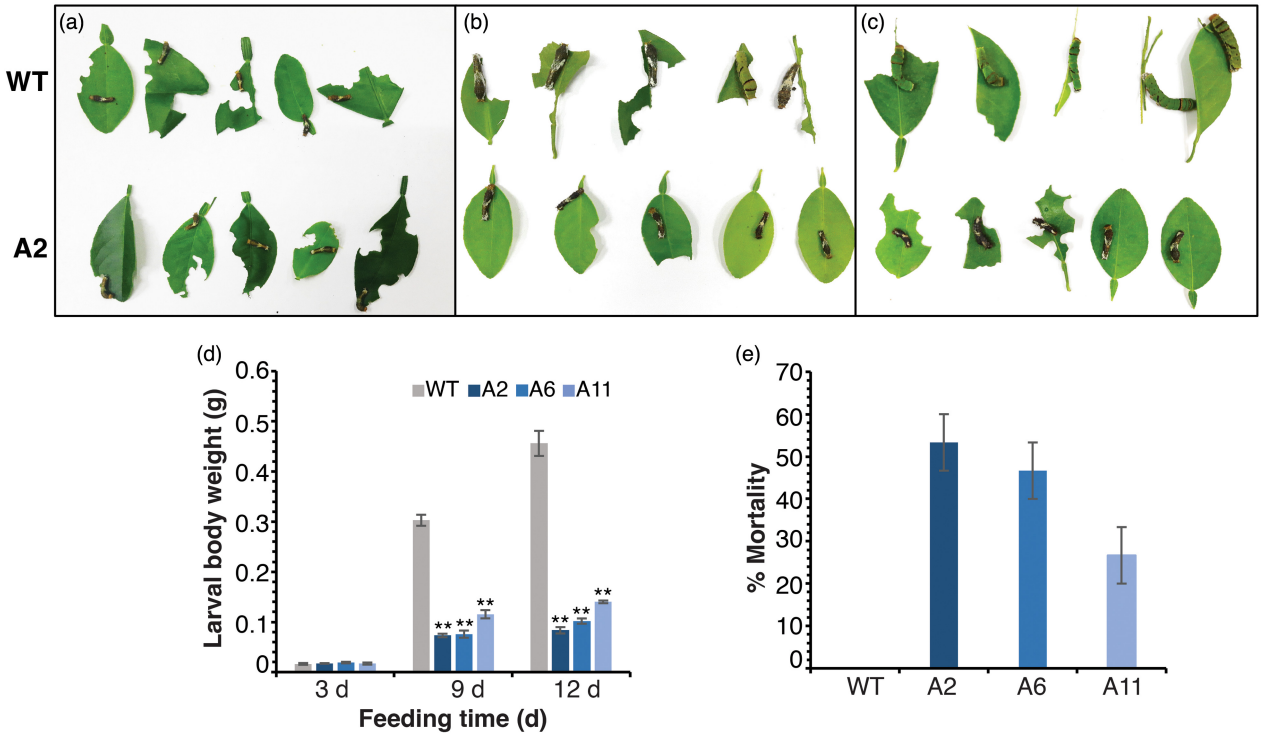

Figure 5 *PRpnp* overexpression enhances resistance against *Papilio demoleus* larvae. WT and transgenic leaves (plant A2, A6 and A11) were fed to five-second instar larvae. Infestation of the larvae at (a) 4 day-post-hatching (DPH), (b) 9 DPH and (c) 12 DPH. Transgenic-fed larvae showed significantly visible retarded growth with delayed instar development between 9 and 12 DPH. (d) A comparison of body weights at the indicated time and (e) mortality (%) at 12 DPH of WT- and transgenic-fed larvae. Lower body weights and increased mortality of transgenic-fed larvae signify enhanced resistance in transgenic plants. Data represent means ± SE (n = 3) of three biologically independent experiments. The asterisks represent a significant difference between WT and transgenic lines were calculated using Student's t-test, **P*-value <0.05 and ***P*-value <0.01. For representation, a comparative larval infestation on WT and transgenic leaves A2 is shown.

and transcription factors (TFs) (*PRXIIF*, *MES1*, *HSP90-5*, *HSFA4A*, *DTX35*, *APX1*, *BZIP60*, *BHLH130*, *NAC52*, *WRKY75*), cell cycle (*CDC6B*, *APC7*, *SPC25*), DNA repair (*PAXIP1*, *MLH3*, *DRT111*, *RAD50*, *LIG6*), transporters (*ABCG40*, *ABCB25*, *ABCC3*, *ECA3*, *HAK5*, *CAT5*), photosynthesis (*PHOT1B*, *LHCB4.3*, *CIB22*, *psaA*, *SOQ1*), development (*NFS2*, *CTL1*, *HPT1*, *HMGB9*, *RER1*) and metabolism (*SPS4*, *PFK3*, *HXK2*, *HIDM*) were significantly up-regulated (Figure S7a–i). The down-regulated genes belong to categories such as lipid metabolism (*KCS2*, *HHT1*, *CYP86A2*, *FAR3*), cell fate (*LIS*, *YAB5*, *GL3*, *WER*), catabolism (*LAC11*, *PME8*, *XYL1*) and development (*COL9*, *EMF2*, *FIE2*, *CLF*). Genes that negatively regulate hormone signalling (*PP2C51*, *ICMTB*, *ARAC7*, *ATAF1*, *B'GAMMA*, *APT1*) were also down-regulated (Figure S7j–m). Total up-regulated and down-regulated DEGs in *PRpnp* transgenic *C. aurantifolia* are documented in Tables S3 and S4, respectively. The global view of the transcriptomic data showed that even without stress signals, the expression of genes associated with plant defence, growth and development was significantly up-regulated in transgenic plants, suggesting the reason for being more resilient to adverse environmental conditions over WT.

LC/MS–MS analysis validates higher accumulation of endogenous ABA, JA and CK in *PRpnp* transgenic plants

After analysing the transcriptome data, we realized that the genes related to ABA and JA biosynthesis and signalling pathways had been up-regulated. Also, some previous work has claimed the participation of purine metabolism enzymes in CK metabolism (Schoor *et al.*, 2011; Zhang *et al.*, 2013). Since *PRpnp* is a salvage pathway enzyme of purine biosynthesis (Verma *et al.*, 2017, 2022). Thus, we anticipated the role of *PRpnp* in catalysing CK interconversion reactions. To understand the phytohormonal regulation by overexpression of *PRpnp* in *C. aurantifolia*, we performed a targeted profiling of ABA, JA and CK in WT and transgenic plants by LC–MS/MS under normal growth conditions. Consistent with our transcriptome data and hypothesis, phytohormone quantification revealed that *PRpnp* transgenic *C. aurantifolia* had a substantially higher accumulation of ABA (~1.86-fold), JA (~2.56-fold) and CK-free base tZ (~2.1-fold), compared to WT. The concentration (ng/g FW) in transgenic plants was estimated to be 41.90, 148.73 and 1.05, whereas the concentration in WT was 22.50, 58.67 and 0.48, respectively, for ABA, JA and CK (Figure 7a–c). It seems that *PRpnp* could elevate the expression of ABA- and JA-related genes thus, promotes their co-accumulation and synergistic interaction. *PRpnp* could also be involved in CK metabolism by regulating its interconversion and promoting the accumulation of CK-free bases.

Discussion

Plants are the fundamental pillars for sustaining a balanced ecosystem for all life forms; thus, advanced sequencing

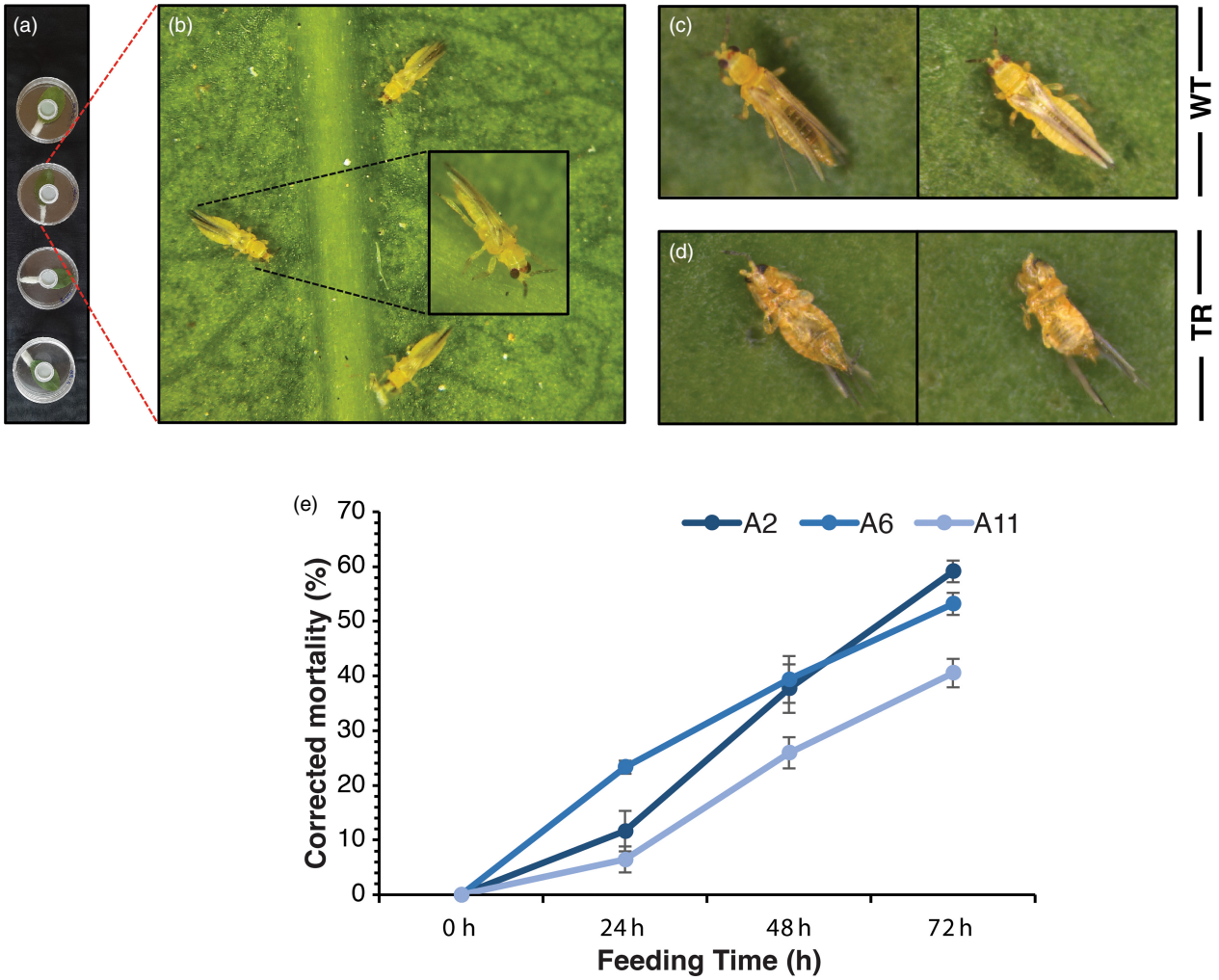

Figure 6 *PRpnp* overexpression enhances resistance against adult *Scirtothrips citri*. Adult thrips collected from the infected plant were fed to prepared WT and transgenic leaves and corrected mortality; CM (%) was monitored for 72 h. (a) Experimental setup for bioassay. (b) Microscopic image of *S. citri* infestation on the detached leaf. Inset—zoomed image of an adult thrips. Photographs of (c) healthy and (d) dead thrips fed on WT and transgenic (TR) leaves, respectively. (e) Increased CM (%) of adult *S. citri* in A2, A6 and A11 represents enhanced resistance in *PRpnp* transgenic plants. Data represent means ± SE (n = 3) of three biologically independent experiments.

techniques frequently upgrade the plant genome and proteome databases. However, the roles of several plant genes and proteins remain undiscovered. Similarly, one 'yet to be studied' group of proteins is related to WI protein and VSPs and shows homology to nucleoside phosphorylases. In our previous studies, we characterized one of the proteins from this group, that is, PRpnp and proved its neofunctionalization to a TI (Chaudhary *et al.*, 2008; Verma *et al.*, 2017, 2022). We speculated *PRpnp* as a potential candidate that can promote multiple stress tolerance in plants. Since it is a purine salvage enzyme and no other studies have shown the defensive role of *PRpnp* or other members of this protein group. Thus, we have overexpressed *PRpnp* in *C. aurantifolia* to extend our knowledge and understanding of the importance of this novel PNP family protein.

We determined the subcellular localization of PRpnp to decode its function. In this study, PRpnp was co-localized in the nucleus and cytosol upon transient expression of PRpnp-GFP fusion protein in *N. benthamiana* by agro-infiltration (Figure 1b). Previously, few studies unveiling the localization of mammalian PNPs have been documented; although, to our best knowledge, no such reports on plant PNPs are available. The reported PNPs are cytosolic enzymes (Giuliani *et al.*, 2017; Rubio and Berne, 1980), though nuclear-cytoplasmic (Castellano *et al.*, 1990), mitochondrial (Haag and Lewis, 1994) and extracellular PNPs (Giuliani *et al.*, 2017) are also present. As purine nucleotides are crucial for nucleic acid generation, providing cellular energy (ATP), intracellular signalling (GTP) and also serve as cofactors such as NAD and coenzyme A (Pedley and Benkovic, 2017), thus all the dividing, differentiating and fully differentiated cells seek a vast supply of purines throughout life (Giuliani *et al.*, 2017). Inside the cell, purine nucleotides control the G_1 to S phase transition and progression through the S phase (Quéméneur *et al.*, 2003). In a study, the growth rate of cultured fibroblasts increased upon increasing the supply of purine nucleotides due to the promotion of the G1/S transition and ATP production (Kondo *et al.*, 2000). Purine metabolites are also involved in DNA repair by promoting the repair of double-strand breaks (Zhou *et al.*, 2020). Consistent with these findings, the nuclear-cytoplasmic localization of PRpnp

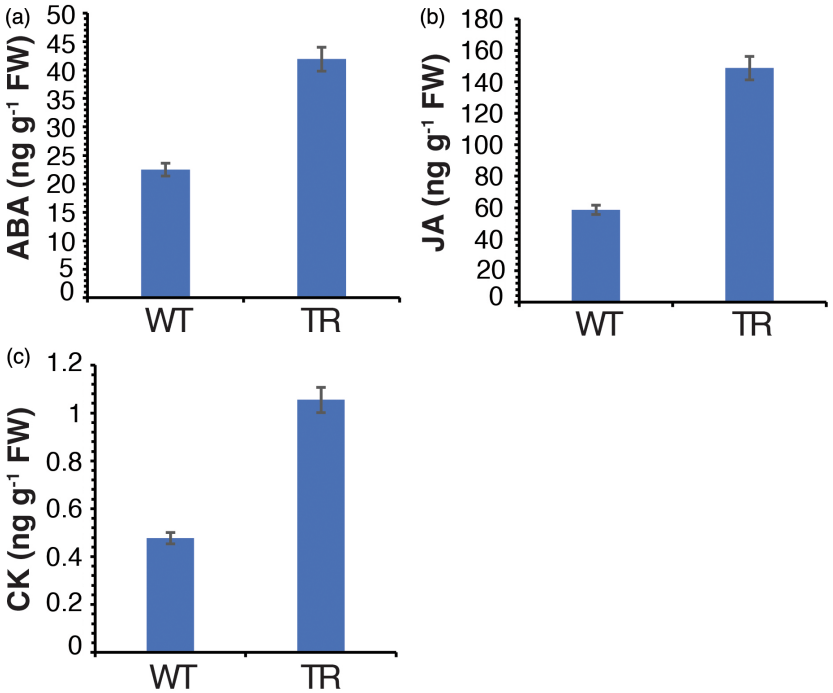

Figure 7 Phytohormones quantification by LC–MS/MS in WT and *PRpnp* transgenic plants. (a) abscisic acid (ABA), (b) jasmonic acid (JA) and (c) cytokinin (CK; tZ). *PRpnp* overexpression caused more endogenous accumulation of tested phytohormones under normal growth conditions. Measurements are averaged over three replicates. Error bars represent ± SE.

corroborates that it could regulate several biological processes in the nucleus and cytosol by maintaining the intracellular purine reservoir. *PRpnp* could also promote nucleic acid synthesis, DNA replication and even DNA repair by fulfilling the need of purine nucleotides in the nucleus. However, the mechanism of nuclear transport of PNPs remained unclear.

After abiotic stress treatment, *PRpnp* transgenic plants showed significantly higher retention of chl a, chl b and carotenoids in leaf disc senescence assays that signifies their enhanced tolerance against salt, oxidative and alkaline pH stress, compared to WT (Figure 2). Previous reports are available showing the reduction in the concentration of chlorophyll and carotenoid under the influence of several stresses (Hamani *et al.*, 2020; Sidhu *et al.*, 2017). Further, *PRpnp* overexpressing plants also showed salt tolerance under soil conditions (Figure 3) and drought tolerance (Figure 4). To reveal the underlying mechanism that leads to multiple stress tolerance in Mexican lime by overexpressing *PRpnp*, we performed *de novo* transcriptome profiling of WT and transgenic plants, under normal growth conditions. We found a comprehensive set of genes involved in phytohormones majorly, ABA and JA biosynthesis and signalling, plant defence, cell cycle, DNA repair, TFs, transporters, development and metabolism were up-regulated in transgenic plants (Figure S7).

Phytohormones are the steward of plant defence and responses. They coordinate the environmental signals with developmental processes and are the basis of stress endurance in plants (Altmann *et al.*, 2020). Several essential genes related to ABA such as biosynthesis (*AAO3*), cofactor (*ABA3*), TFs (*ABF2*, *BZIP16*, *BZIP60*, *BHLH130*, *NAC52*), other positive regulators (*ARIA*), ABA importers (*ABCG40*) and receptors (*PYL9*) were differentially up-regulated (Figure S7a,c,d). Moreover, a negative regulator of ABA-mediated responses, protein phosphatase 2C clade A member (*PP2C51*), was differentially down-regulated in *PRpnp* transgenic plants (Figure S7j). Similarly, JA biosynthesis (*LOX6*, *OPR3*, *AOC3*, *4CLL5*, *4CLL7*, *4CLL9*, *KAT2*) and positive regulators (*WRKY75*, *PLA1*) were also up-regulated in transgenic plants (Figure S7a,c). Previous studies have elucidated the importance of these genes in phytohormone-mediated signalling and their role in improving stress resistance in plants (Tables 1 and 2). Through the transcriptome data, we anticipated that even without stress signals, *PRpnp* transgenic plants could accumulate higher levels of endogenous ABA and JA, relative to WT plants. Our LC–MS/MS data confirmed this and quantified higher levels of both phytohormones in transgenic plants (Figure 7).

Literature that shows, under natural conditions, plants protect themselves by elevating endogenous ABA (Vishwakarma *et al.*, 2017) and JA (Kim *et al.*, 2021) is available which in turn increases transcript levels of WI protein (Moura and Ryan, 2001) or VSPs (Avice *et al.*, 2003) or TIs (Srinivasan *et al.*, 2009), indicating their prominent role in plant stress tolerance. In agreement with this, overexpression of WI proteins (Medeiros *et al.*, 2012), VSPs (Abbaraju *et al.*, 2022) and TIs (Srinivasan *et al.*, 2009) enhances abiotic and biotic stress tolerance; however, the mechanism behind improved stress resistance in transgenic plants are still unknown. In our study, we found that upon overexpression, *PRpnp* elevated the endogenous accumulation of ABA and JA which is responsible for the resistant phenotypes in Mexican lime. This suggests that *PRpnp* overexpression might be involved in some regulatory mechanism or produces signals that trigger the transgenic Mexican lime to produce 'stress fighting' phytohormones more than the usual level. From this, we can conclude that, when overexpressed,

Table 1 Differentially regulated genes in *PRpnp* transgenic *Citrus aurantifolia* related to ABA biosynthesis and signalling

Gene	Description	F.C	Reference
AAO3	Abscisic-aldehyde oxidase	2.98	Seo *et al.* (2000)
ABA3	Molybdenum cofactor sulfurase	5.17	Bittner *et al.* (2001)
ARIA	Arm repeat protein interacting with ABF2	10.52	Kim *et al.* (2004)
NAC052	NAC domain-containing protein 52	8.50	Gao *et al.* (2010)
ABCG40	ABC transporter G family member 40	3.14	Kang *et al.* (2010)
BZIP16	bZIP transcription factor 16	3.33	Chen *et al.* (2012)
BZIP60	bZIP transcription factor 60	6.36	Zhang *et al.* (2015)
PYL9	Abscisic acid receptor PYL9	5.41	Zhao *et al.* (2015b)
AREB3	Abscisic acid-insensitive 5-like protein 2	10.21	Wang *et al.* (2016)
PP2C51	Protein phosphatase 2C 51	−6.60	Bhatnagar *et al.* (2017)
BHLH130	Transcription factor bHLH130	3.78	Zhao *et al.* (2020)

Table 2 Differentially regulated genes in *PRpnp* transgenic *Citrus aurantifolia* related to JA biosynthesis and signalling

Gene	Description	F.C	Reference
KAT2	3-ketoacyl-CoA thiolase 2	5.33	Afitlhile *et al.* (2005)
4CLL5 (*OPCL1*)	OPC-8:0-CoA ligase 1	11.50	Koo *et al.* (2006)
4CLL7	4-coumarate--CoA ligase-like 7	7.81	
4CLL9	4-coumarate--CoA ligase-like 9	3.66	
PLA1	Phospholipase A I	9.93	Yang *et al.* (2007)
AOC3	Allene oxide cyclase 3	4.43	Gu *et al.* (2012)
LOX6	Lipoxygenase 6	5.61	Chauvin *et al.* (2013)
OPR3	12-oxophytodienoate reductase 3	7.17	Pigolev *et al.* (2018)
WRKY75	Probable WRKY transcription factor 75	2.69	Chen *et al.* (2021)

other similar proteins such as WI protein, VSPs or TIs could also increase the basal levels of defence hormones such as ABA or JA which enhances stress resilience in plants. The elevated phytohormone in turn could increase the expression of WI proteins, VSPs, TIs and other stress-responsive genes. Additionally, over time, *PRpnp* evolved and attained an additional trypsin inhibitory property. The fundamental function of TIs is to prevent the degradation of the total protein content of the cell by blocking the proteases (Srinivasan *et al.*, 2009; Srinivasan and Kirti, 2012). It seems that under natural stress conditions, *PRpnp* might be involved in the suppression of proteases that would improve the stability of various stress-responsive proteins and enhances basal plant immunity. Thus, *PRpnp* transgenic plants must be superior to WT in having a ceaseless and undegraded protein pool. Collectively, *PRpnp* not only elevates ABA and JA levels but also improves the survival of other stress-responsive proteins thus, improves plant vigour and stress resistance in transgenic plants.

From the parallel accumulation of ABA and JA we conclude that upon stress perception, both the signalling pathways would work synergistically, complementing each other responses including other metabolic and signalling pathways in response to abiotic stresses in transgenic *C. aurantifolia*. Evidence showing the existence of synergism between ABA and JA in the regulation of the plant response to abiotic and biotic stress are available (de Ollas *et al.*, 2015; Kim *et al.*, 2021; Li *et al.*, 2022). Moreover, the increased proline accumulation and RWC (Figures 3b and 4b,d) and decreased MDA levels (Figures 3c and 4c) in transgenic *C. aurantifolia* upon stress treatments, relative to WT are consistent with previous reports that show similar results (Cui *et al.*, 2011; Ma *et al.*, 2020). Therefore, the increased proline and RWC may have shielded the antioxidative enzymes and excess water loss, respectively and low MDA levels prevented membrane damage in transgenic Mexican lime thus, alleviating the deleterious impacts of salt and drought stress.

The improved plant vigour of *PRpnp* transgenic plants was evident by upregulation of several important plant defence and TFs genes/families that protect plants from stresses and majorly belong to peroxiredoxins, heat shock protein and heat stress TFs, L-ascorbate peroxidases, cysteine-rich receptor-like kinases (CRKs), G-type lectin S-receptor-like serine/threonine protein kinase (GsSRK), bZIP and WRKY family (Figure S7b,c). In agreement with the studies that reveal the importance of purine nucleotides in the G_1 to S phase transition (Quéméneur *et al.*, 2003), progression through the S phase (Kondo *et al.*, 2000) and DNA repair (Zhou *et al.*, 2020), our transcriptome data, showed the up-regulation of a number of gene sets that positively regulates cell cycle and DNA repair in *PRpnp* transgenic plants (Figure S7e,f). We also found multiple gene sets involved in photosynthesis, especially those related to photosynthetic electron transport such as photosystem II (PSII), photosystem I (PSI) and ATP synthase were up-regulated in transgenic plants (Figure S7g). This highly intact photosynthetic machinery suggests that it could be one of the major factors leading to higher pigment retention in transgenic plants during leaf disc senescence assay.

PIs such as TIs are shown to significantly impair the growth of Lepidopteran larvae (Srinivasan *et al.*, 2009). Intake of TI along with food inhibits nutrient absorption and produces a feed inhibition signal which ultimately causes insect death (Zhao *et al.*, 2019). Therefore, *PRpnp* was expected to improve insect resistance. Consistently, the feeding assay conducted with *P. demoleus* larvae showed significantly decreased body weights and increased mortality rates of transgenic-fed larvae in comparison to the control (Figure 5d,e). This caused due to the inhibition of digestive proteases of *P. demoleus* larvae, which is majorly trypsin. The inhibited proteolytic process by PRpnp led to the unavailability of essential amino acids and caused physiological stress followed by retarded insect growth and death.

The proteolytic activity in the whole extracts of *Frankliniella occidentalis*, a thrips that belongs to the same family as *S. citri* is predominantly cysteine proteases (Annadana *et al.*, 2002). Later, Kuipers and Jongsma (2004) also reported cysteine proteases as prime digestive enzymes in thrips gut. Though *PRpnp* is a trypsin protease inhibitor, we apprehended that it might not have a direct role in thrips resistance. However, in several previous reports, JA restricted thrips performance and preference, and also improved resistance (Abe *et al.*, 2008, 2009). A dramatic increase in thrips attraction was observed in non-infected JA-insensitive *coi1-1* mutants compared with WT plants (Abe *et al.*, 2009). Exogenous application of methyl jasmonate restored the thrips repellency effect in tomato mutants *def-1* which was impaired in JA biosynthesis (Escobar-Bravo *et al.*, 2017). We also found increased mortality in transgenic-fed adult *S. citri*, compared to

control (Figure 6d). From this, we conclude that the increased JA levels due to *PRpnp* overexpression in Mexican lime enhanced thrips resistance.

Naturally occurring CKs are N^6 derivatives of adenine (Frebort *et al*., 2011). Among all the cytokinin metabolic processes, its interconversion plays a pivotal role in maintaining CK homeostasis. The purine salvage enzymes are known to catalyse the interconversions of CK bases, ribosides and nucleotides (Ashihara *et al*., 2018). So, we anticipated *PRpnp*, an enzyme of the purine salvage pathway could be involved in CK metabolism. We detected a higher level of endogenous CK-free base; tZ in *PRpnp* transgenic plants by LC–MS/MS (Figure 7c). Thus, *PRpnp* plays an important role in promoting CK interconversions, positively regulating the level of active CK bases and thus maintaining its homeostasis.

Collectively, in this study, we constitutively overexpressed PRpnp, a novel PNP family protein in *C. aurantifolia* Cv. Pramilini and obtained transgenic plants with enhanced tolerance to various abiotic and biotic stresses. Moreover, our results indicated the mechanism by which overexpression of *PRpnp* enhanced stress tolerance in transgenic Mexican lime that involves increasing the endogenous levels of ABA and JA and promoting their synergistic interactions. We also found other important functions of PRpnp including the maintenance of intracellular purine reservoir, CK homeostasis and improving plant vigour.

Experimental procedures

Subcellular localization of PRpnp

For subcellular localization, *PRpnp-GFP* fusion cassette was made under the control of the constitutive CaMV 35S promoter (35S::PRpnp-GFP). *PRpnp* gene was amplified without stop codon and cloned into the entry vector, pDONR221. The entry clone was then shuttled to the destination vector pB7FWG2 by Gateway technology (Karimi *et al*., 2002). Overnight grown *Agrobacterium* strain GV3101 harbouring pB7FWG2-35S::PRpnp-GFP vector was infiltrated into the abaxial surface of 4-week-old leaves of *N. benthamiana*. For constitutive expression of RFP, vector pK7WG2-RFP was infiltrated. Live-cell imaging using laser scanning confocal microscopy was performed with a Leica TCS SP8 (Leica Microsystems, Wetzlar, Germany) and the images were processed with software LAS-AF (Version 4.0.0.11706).

Construction of overexpression vector

For generating the *PRpnp* overexpression construct, CaMV 35S promoter (enhanced), *PRpnp* (Accession number: HQ332518) and T-nos were assembled in pBSSK(+) and then subcloned into binary vector pCAMBIA2301 to generate the pCAMBIA2301-2x35S::PRpnp construct. Hypervirulent *Agrobacterium* strain EHA105 was used for plant transformation. All the plasmids used in the study were confirmed by sequencing before proceeding. The primers and PCR conditions are documented in Tables S5 and S6, respectively.

Plant material preparation

Sterilized mature seeds of *C. aurantifolia* Cv. Pramalini were germinated on SG media for 3–4 weeks at 25 ± 2 °C in dark, followed by 1 week under a 16-h photoperiod. Light green epicotyls of ~2 cm^2 length with oblique cut ends were pre-treated in 60 mL of LM2NB media for ~3–4 h and used for transformation.

Agrobacterium-mediated transformation and regeneration

Agrobacterium strain EHA105 harbouring binary vector pCAMBIA2301-2x35S::PRpnp was revived on YEM agar. One loop of the culture was inoculated to yield an OD_{600} of 0.3 into 60 mL of YEM broth on the day of transformation with appropriate antibiotics and 100 μM acetosyringone. The *Agrobacterium* cells were centrifuged and resuspended in MSA (OD_{600} = 0.3). The pre-treated epicotyls were infected by *Agrobacterium* for 15 min and blotted dry on sterile Whatman filter paper. Infected epicotyls were co-cultivated and regenerated, respectively on SMA2NB media for 2 days and MRNB1 media for 4 weeks at 25 ± 2 °C in dark and later shifted to a 16-h photoperiod for one additional week.

Screening and hardening of putative transgenic plants

The basal portion of regenerated shoots was tested histochemically for GUS activity (Jefferson *et al*., 1987). Transgenic plants were recovered either by rooting or *in vitro* micrografting. For rooting, the GUS-positive shoots were cultured on MR media whereas, for *in vitro* micrografting, the scion-rootstock system was cultured on ML media. GUS negative shoots were discarded. Recovered transgenic plants were slowly acclimated to the greenhouse conditions. All the media compositions are documented in Table S7.

Confirmation of putative transgenic plants

The total genomic DNA was isolated from the leaves of WT and putative transgenic plants using DNeasy® plant mini kit (69 104; Qiagen, Hilden, Germany) according to the manufacturer's protocol and quantified with NanoDrop microvolume UV–Vis spectrophotometers (ND-ONE-W; Thermo Fisher Scientific, Waltham, MA). To confirm the stable transgenic plants, we performed duplex PCR and amplified *PRpnp* and *nptII* using the gene-specific primers and genomic DNA.

RNA extraction and transcript analysis

Leaves of WT and PCR-positive transgenic plants were used to extract the total RNA with RNeasy® plant mini kit (74 904; Qiagen) as per the manufacturer's instructions and was quantified. First-strand cDNA was synthesized (1 708 841; Bio-Rad, Hercules, CA), and the qRT-PCR assay was performed in 96-well optical plates using SYBR Green dye (A25741; Applied Biosystems™, Waltham, MA) and QuantStudio™ 5 real-time PCR system (A28574; Applied Biosystems™). QuantStudio™ Design and Analysis Desktop Software v1.5.1 was used for analysis. The expression of the *PRpnp* gene in transgenic plants was confirmed by comparing its cycle threshold (C_t) value with WT plants. The relative quantification of the *PRpnp* gene was normalized to the cytochrome oxidase gene (*cox*) and calculation was done by comparative C_T method (Livak and Schmittgen, 2001).

Western blot analysis

The total leaf protein was isolated (Omar *et al*., 2007) and quantified (Bradford, 1976) as described previously and then transferred to the PVDF membrane (IPVH00010 Immobilon®-P; MilliporeSigma™, Burlington, NJ). After blocking, the membrane was first incubated with an anti-PRpnp antibody (1:1000) followed by anti-rabbit IgG, HRP-linked antibody (1:2000) (7074P2; Cell Signaling Technology, Danvers, MA). The protein

blots were detected by Pierce™ ECL Western blotting substrate (32 109; Thermo Fisher Scientific) as per the manufacturer's protocol using ChemiDoc™ (1 708 265; Bio-Rad).

Analysis of PNP and trypsin inhibitory activities

The total protein was isolated and tested for PNP (Verma *et al*., 2017) and trypsin inhibitory activity (Yao *et al*., 2001) as described earlier.

Leaf disc senescence assay for multiple abiotic stress resistance

The leaf discs of 1 cm diameter were excised from 18–20 months old plants. An equal number of leaf discs ($n = 12$) were floated in 15 mL of ½ strength Hoagland solution supplemented with NaCl (200 and 300 mM; 48 and 72 h) and H_2O_2 (50 mM; 48 h) for salt and oxidative stress, respectively. For alkaline stress, leaf discs were dipped in 15 mL of ½ strength Hoagland solution of pH = 9 for 120 h. An equal volume of ½ strength Hoagland solution (pH = 5.8) without any supplements was used for the control experiment. Leaf discs were incubated at 25 ± 2 °C under continuous white light. Chlorophyll and carotenoids were quantified as already described (Hiscox and Israelstam, 1979).

Analysis of salt stress tolerance at the whole plant level

The 18–20 months old plants were irrigated twice a week for 45 days with an equal amount of ¼ strength Hoagland solution supplemented with 75 mM NaCl. To minimize the environmental variations, the positions of pots in the greenhouse were interchanged daily. The free proline and MDA content were estimated at regular intervals.

Analysis of drought stress tolerance

The 18–20 months old were assessed for drought stress tolerance. To evaluate the drought tolerance, water supply to plants was withheld for 25 days. The same environmental condition was given to every plant. Plants were photographed and proline levels, MDA content and RWC estimations were done at different time points. After the drought stress period, the plants were reirrigated and evaluated for survival after 15 days.

Quantification of proline, MDA and RWC

The free proline content was calculated as described previously (Bates *et al*., 1973). Lipid peroxidation was estimated by quantifying the MDA level following the method described by Hodges *et al*. (1999). The MDA concentration produced in the sample was estimated by the equation described by Jha *et al*. (2013). RWC was estimated as already documented (Morgan, 1984).

Detached leaf bioassay for evaluating resistance to *P. demoleus* larvae

Tender leaves were fed to second instar larvae ($n = 5$) of *P. demoleus*. The insects were kept at 25 °C under a 16-h photoperiod with 70% relative humidity and larval body weight was recorded at regular intervals. The mortality rate of the larvae was calculated at 12 DPH using the formula described earlier (Zubair *et al*., 2019).

Detached leaf bioassay for evaluating resistance to adult *S. citri*

For detached leaf bioassay, young and tender leaves were wrapped in cotton and sealed with Parafilm. A small cut was made in the sealed portion to add water droplets periodically to prevent leaf drying. The thrips were cultured at 30 °C in a 16-h photoperiod. To determine the mortality rate, the *S. citri* were counted manually using a double-lens magnifying glass at different time points and imaged using a stereomicroscope (M205 A; Leica, Wetzlar, Germany). Mortality data were corrected using Abbott's formula (Abbott, 1925).

Transcriptome profiling and analysis

Total RNA was isolated from WT leaves and *PRpnp* transgenic plants as described earlier and used for *de novo* transcriptome profiling. Two biological replicates were used for the analysis, and RNA-Sequencing was performed using the Illumina HiSeq platform with a paired end read length of 101 bp at Bionivid Technology Private Limited (Bengaluru, India). NGSQC toolkit was utilized to check the quality (Phred score > 20) of raw reads (https://github.com/mjain-lab/NGSQCToolkit, Patel and Jain, 2012). *De novo* assembly and mapping were performed using Trinity software (https://github.com/trinityrnaseq/trinityrnaseq, Haas *et al*., 2013). The abundance estimation was calculated using RNA-Seq by Expectation–Maximization (RSEM) method (Li and Dewey, 2011). The differential gene expression analysis was performed using DESeq2 (https://bioconductor.org/packages/release/bioc/html/DESeq2.html, Love *et al*., 2014). Transcripts having log2 fold change greater than or equal to 2 were considered as DEGs. The functional annotation of the transcriptome was performed using the Trinotate pipeline (https://github.com/Trinotate/Trinotate.github.io/blob/master/index.asciidoc), employing uniport-Swissprot (https://ftp.uniprot.org/pub/databases/uniprot/current_release/knowledgebase/complete/) database for blastx and blastp searches. Cytoscape (v 3.9.1) plug-in bingo was used to perform GO enrichment and network analysis (Maere *et al*., 2005). Further, heat maps were constructed using gplots and RColorBrewer R packages (https://CRAN.R-project.org/package=gplots, Warnes *et al*., 2022; https://CRAN.R-project.org/package=RColorBrewer, Neuwirth and Neuwirth, 2014). PCA and volcano plots were constructed using python.

ABA and JA quantification

Briefly, around 200–250 mg of fresh tissues were grounded in liquid nitrogen. Methanol (1 mL) and internal standard (IS; 4 μL) were added to each sample followed by centrifugation. The samples were dried using SpeedVac concentrator (Savant SPD1010; Thermo Fisher Scientific). The methanol (400 μL) reconstituted samples were subjected to LC–MS/MS (QTRAP 6500+; SCIEX, Framingham, MA) for ABA and JA estimation.

CK quantification

Fresh plant tissue (200–250 mg) was grounded in liquid nitrogen and extracted in extraction buffer (1 mL; methanol:water:formic acid; 15 : 4 : 0.1) with 10 μL of IS. The sample was centrifuged and the collected supernatant was purified with a C_{18} RP SPE column and eluted in 0.1% formic acid in acetonitrile. The samples were dried using SpeedVac concentrator and reconstituted in 100 μL of 5% methanol and subjected to LC–MS/MS.

Statistical analyses

All the data in the study are generated by three independent experiments with three replicates. Statistical analyses of the data were performed in Microsoft Office Excel 2019. Error bars in the graphs are standard errors of the mean (±SE). Experimental data were subjected to Student's *t*-test analyses to determine the

statistical significance between WT and *PRpnp* transgenic plants or between control and treatment sets.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

SS and HC conceived the project and designed the experiments. SS performed most of the experiments and analysed the data; SG and HG performed subcellular localization; CK and SS analysed the RNA-sequencing data; CK, LV and JG conducted and analysed the LC–MS/MS experiments; RDB and GTB performed thrips mortality bioassay; ZT assisted in recombinant protein purification for antibody generation; DKS gave substantial suggestions during experiments; SS wrote the manuscript; HC and AKS revised the manuscript. All authors read and approved the manuscript.

Data availability statement

RNA-seq datasets related to this work have been deposited in Gene Expression Omnibus (GEO) with the accession number GSE208104.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Confirmation of molecular cloning of expression cassette in pBSSK(+) cloning vector and pCAMBIA2301 binary vector and vector construct of pCAMBIA2301-2x35S::PRpnp.
Figure S2 Procedure of development of transgenic *Citrus aurantifolia* Cv. Pramalini overexpressing *PRpnp* by *Agrobacterium*-mediated transformation.
Figure S3 Molecular characterization of putative *PRpnp* transgenic plants by duplex PCR and qRT-PCR.
Figure S4 Characterization of transgenic *Citrus aurantifolia* by Western blot analysis and assessment of PRpnp dual activity.
Figure S5 (a) PCA and (b) volcano plot of transcriptome profiles of WT and *PRpnp* transgenic *Citrus aurantifolia* with two replicates under normal growth conditions.
Figure S6 Network analysis showing the enriched pathways of the up-regulated (a–e) and down-regulated (f–h) DEGs.
Figure S7 Heat map showing the hierarchical clustering of the up-regulated (a–i) and down-regulated (j–m) DEGs.
Table S1 Total recovered GUS-positive transgenic *Citrus aurantifolia* plants.
Table S2 *De novo* transcriptome summary.
Table S3 List of up-regulated DEGs.
Table S4 List of down-regulated DEGs.
Table S5 List of primers.
Table S6 PCR conditions.
Table S7 Culture media composition.