

PRIDA, a protoplast-based assay for efficient identification of oxidative- and heat-stress regulatory genes

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Method Article

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Abstract

Background: Rapidly identifying candidate genes potentially involved in stress tolerance is crucial for functional genetic studies in non-model plant species. *Agrobacterium*-based transient assays can be conducted in many plants using fleshy tissues such as leaf/fruit/petal. However, such assays are not feasible in perennial ryegrass (*Lolium perenne* L.) and most other grass species since these plants have no fleshy tissue to be readily infected.

Results: In this study, we developed a **P**rotoplast-based **R**apid stress regulatory gene **I**dentification **A**ssay (briefed as 'PRIDA') to rapidly identify genes in the regulation of plant heat and oxidative tolerance in perennial ryegrass. Using ryegrass mesophyll protoplasts, vectors harboring target genes or their RNAi hairpin structures driven under the maize ubiquitin promoter were transformed into protoplasts to transiently over-express or suppress the target genes. The transformed protoplasts were then subjected to optimized heat (35 to 37 °C) or oxidative (50 to 75 mM H₂O₂) stress, and their viabilities were immediately recorded under a microscope after 0.1% Evans blue dye staining. The validity and reliability of PRIDA were verified by transiently knocking-down *LpNOL/LpSGR* and over-expressing *LpTT3.1/LpTT3.2* that led to significantly compromised or enhanced protoplast viability after heat or H₂O₂-induced oxidative stresses. Finally, using 'PRIDA', we identified three heat-induced differentially expressed *E3 ligase* genes as potential regulators in plant heat and/or oxidative stress tolerance.

Conclusion: This protoplast-based 'PRIDA' can be used as a versatile assay to rapidly identify and characterize candidate regulatory genes in heat and oxidative stress that will accelerate molecular genetic studies in perennial ryegrass. This assay can be adapted to other non-model plant species after adjusting abiotic stress parameters for further research and the development of improved cultivars.

Background

In reverse genetics studies, characterization of the target gene's molecular functions often requires an analysis of the plant's phenotypic alterations due to over-expressing or knocking-out/down the target gene(s). However, in many non-model plant species, the laborious process of generation of stable transgenic plants alone takes more than six months and remains challenging to most novices, not to mention that there is no genetic transformation system in some agronomic-important plant species or specific varieties. To complement these limitations of stable genetic transformations, various transient assays were developed and applied in plant molecular genetics. For example, *Agrobacterium*-mediated or biolistic-based transient gene expression was applied in some non-model plant species to analyze the target gene's effect on color formation, senescence, and many other developmental processes in fleshy tissues such as leaves, fruits, and petals (Spolaore et al. 2001; Pi et al. 2019; Colinas and Goossens 2022; Hussein et al. 2013; Shang et al. 2007; Krenek et al. 2015). Virus-induced gene silencing (VIGS) systems were developed in some dicot and certain grass species for gene silencing, overexpression [virus-induced overexpression (VOX)], genome editing [virus-induced genome editing (VIGE)], and host-induced gene silencing (HIGS) in leaves and fruits (Rössner et al. 2022). Compared to the time-consuming process of

stable genetic transformation, these transient gene expression systems can be done within days, not to mention the time saved for flower and fruit sets in non-model plant species. Yet, these VIGS-based systems were still limited to a few plant species mainly due to the limitation of maneuverable virus and amenable host species (Rössner et al. 2022), and the *Agrobacterium*-mediated or biolistic-based transient gene expression is typically effective on fleshy tissues, such as fruits, petals, and dicot leaves. For many grass species in the Poaceae family, the abovementioned transient assays were still not applicable.

Mesophyll protoplast-based transient gene expression is another alternative for functional analysis of target genes, as the freshly isolated protoplasts keep highly similar physiological characteristics of whole leaves (Yoo et al. 2007). Currently, protoplast-based transient gene expression has been routinely used for protein subcellular localization and trafficking assays, protein-protein interaction analysis by bimolecular fluorescence complementation (BiFC) or firefly luciferase complementation (FLC), co-immunoprecipitation (Co-IP), promoter activation assay, activities of transcription factors and protein kinases, protein stabilities, transcription activation assay by co-transforming the transcription factor (as the effector) and a reporter gene [e.g., *Luciferase (LUC)* gene] driven under specific promoter, etc. (Liu and Xiong 2022). Using the *LUC* reporter driven under a stress-responsive promoter, changes in LUC signal can be readily detected in *Arabidopsis* protoplasts upon stress signals (Confraria and Baena-González 2016). These protoplast-based experiments are highly efficient and relatively facile that most of them can be completed within one or two days. Furthermore, hundreds of transformed protoplasts can be observed within one scope that helps to assure the reliability and reproducibility of experimental results.

Perennial ryegrass (*Lolium perenne* L.) is an important perennial cool-season grass species that is the main fodder source in the temperate world accounting for about 70–80% of the world's cow's milk, beef, and veal production (Wilkins and Humphreys 2003). However, in southern and southwestern China where it is widely used as the primary grass species of grasslands, nearly 50% or more perennial ryegrass cannot survive through the prolonged summer heat. With the continually elevated air temperature, improving its heat-tolerance is one major breeding goal for ryegrass genetic improvement. Identifying regulatory genes in heat tolerance is the basis for plant molecular breeding. Currently, stable genetic transformation of ryegrass takes at least seven months starting from callus initiation to regeneration of putative transgenic plants (Grogg et al. 2022). With the accumulated information of potential heat-responsive genes or genetic loci using comparative transcriptome analysis or Genome-Wide Association Studies (GWAS) (Nie et al. 2022; Zhang et al. 2020), an efficient and time-saving method is in need to rapidly pinpoint out candidate regulatory genes in ryegrass heat tolerance.

The slim leaves of ryegrass are of parallel veins and covered with shining-waxy cuticles making it not amenable to *Agrobacterium* or biolistic-based transient assays. Previously, we developed an efficient mesophyll protoplast isolation and transformation protocol using ryegrass leaves (Yu et al. 2017). We found that these mesophyll protoplasts were susceptible to H₂O₂-induced oxidative stress treatment that could be quantified after Evans blue dye staining for protoplasts with severely damaged plasma membrane under a microscope, and exogenous addition of sodium copper chlorophyllin to the protoplast suspension solution can alleviate the H₂O₂-induced cell death (Yu et al. 2022). Based on this knowledge,

we propose that this protoplast transformation system should be able to tell the transgene's effect on plant heat and oxidative tolerance. In this study, we report the development of the Protoplast-based Rapid stress regulatory gene Identification Assay (we briefed it as 'PRIDA') to rapidly pinpoint out candidate regulatory genes in plant heat and oxidative tolerance.

Methods

Plant material and growth conditions

It is noteworthy that a healthy plant material is the prerequisite for successful protoplast isolation, transformation, and subsequent PRIDA analysis. Perennial ryegrass (cv. Buena vista) was grown in vermiculite: perlite: peat moss (1:3:9) in a growth chamber with temperature set at 25/20°C (day/night), exposed to light 14 hours a day, watered twice a week, and fertilized once a week with half strength Hoagland solution. The middle section of the first fully expanded leaf was used as the material for the isolation of protoplasts.

Reagents and solutions

The formula and preparation procedures of reagents and solutions used in this study were listed in the supplemental data 1–6.

Protoplast isolation and transformation

The procedure of ryegrass mesophyll protoplast isolation and transformation was the same to our previous report (Yu et al. 2017). In brief, the first fully expanded leaves were taken, washed twice, and then wiped off the moisture with absorbent paper. The middle parts of the leaves (~ 0.5 g) were minced into fine fragments of about 0.5-1mm wide in parallel to the leaf vein in a Petri dish containing the enzymatic hydrolysate (supplemental data 1). Then, the petri dishes were vacuumed for 30 min, and gently rotated on a horizontal shaker for enzyme digestion for 4 hours (h) at room temperature. The enzymatic hydrolysate was filtered through a sterilized 75 µl nylon mesh in a 50 ml centrifuge tube with 100×g centrifugation force for 3 min at room temperature. The supernatant was gently removed with a pipette. To each 50ml centrifuge tube, 20 ml of sterilized W5 solution (supplemental data 2) was added to gently resuspend the protoplasts. Then, the protoplasts were precipitated with centrifugation at 100×g for 3 min, resuspended with 15ml sterilized MMG solution (supplemental data 3), and then centrifuged down for another 3 min. After removing the supernatant, the precipitated protoplasts were resuspended in 2 ml of MMG solution and kept on ice before transformation. The density of protoplasts (~ 2×10^5 - 5×10^5 cells per ml) is counted with a hemocytometer under a light microscope (Olympus Model BX53, Tokyo, Japan).

The 1.5 ml centrifuge tubes used for protoplast transformation were rinsed with 1% BSA solution first. Into the tubes, 10 µg plasmids and 100 µl protoplasts were added and gently mixed, then 110 µl of pre-warmed PEG4000 solution (42°C) was added to each centrifuge tube, mixed gently to a homogeneous liquid phase, and stood at room temperature for 20 min. Then, we slowly added 800 µl of W5 solution

along the tube wall to each centrifuge tube, gently mixed it to a homogeneous liquid phase, precipitated it at 100×g centrifugal force for 3 min, and resuspended it with 800 µl W5 solution. The protoplast integrity was detected again under a light microscope. The transformed protoplasts were placed in a 25°C biochemical incubator for 16 h to allow transgene's expression (Yu et al. 2017).

Gene cloning and vector construction

To establish the PRIDA procedure, we used four ryegrass genes in the assay, namely *NON-YELLOW COLOURING 1-like gene* (*LpNOL*) (Yu et al. 2021), *STAYGREEN* or *NON-YELLOWING 1* (*LpSGR*) (Xu et al. 2019), *Thermo-tolerance 3.1* of *L. perenne* (*LpTT3.1*; NCBI Genebank NO.: OR282705) and *LpTT3.2* (Genebank NO.: OR282706), respectively. We used the pVT1629 vector to express the hairpin structure of the target genes for RNA interfering and to over-express the target genes (Xu et al. 2019; Xu et al. 2011). Furthermore, according to transcriptomic data stress (Zhang et al. 2022b; Wang et al. 2017), seven differentially expressed *RING-type E3 ligase* genes under heat stress were cloned into the pVT1629 vector to rapidly characterize their potential regulatory roles in plant heat tolerance using PRIDA.

Heat stress and H₂O₂ treatments on transformed protoplasts

After 16 h of incubation, the transformed protoplasts were ready for heat and oxidative stress treatments. For heat stress, tubes containing protoplasts were transferred to 35 °C (or otherwise at 31°C, 33°C, 35°C, 37°C, 39°C, or 41°C for temperature range test) in water baths for 20 min, stained with 1 µl of 0.1% Evans blue dye for 1 min and then immediately pictured under microscope (supplementary Fig. 2). Three representative fields of view were recorded for counting.

For H₂O₂-induced oxidative stress, 5 µl of H₂O₂ solutions (to the final concentrations of 50, 75, or 100 mM) and 95 µl of the suspended protoplasts were sequentially added into each centrifuge tube, standing for 5 min at room temperature. Then, 1 µL of 0.1% Evan blue solution were added and gently mixed, standing for 1min and then immediately pictured under microscope. Three representative fields of view were recorded for counting. Broken protoplasts stained in blue were counted as died protoplasts and the number of viable ant total protoplasts were artificially counted. Protoplast viability (%) was counted as 'the number of intact protoplasts that are not stained' divided by 'the number of total protoplasts' times 100%.

Statistical analysis

Data were statistically analyzed by One-way ANOVA test at a significance level of 0.05 using SPSS20.0 software (SPSS Inc.). Data are expressed as means ± standard error. At least two separate experiments were carried out for each test to verify the consistency of the results.

Results and Discussion

'PRIDA' is applicable for rapid characterization of heat-stress regulatory genes

In previous studies, we found that stable transgenic ryegrass with suppressed *NON-YELLOW COLOURING 1-like gene* (*LpNOL*) had improved heat tolerance, while the contrary was true for *STAYGREEN* or *NON-YELLOWING 1* (*LpSGR*) (Yu et al. 2021; Yu et al. 2022; Zhang et al. 2022b). To test whether 'PRIDA' was a reliable and sensitive system for characterizing heat-stress regulatory genes, we transfected ryegrass protoplasts with the RNAi constructs of *LpNOL* and *LpSGR* to prove the concept. Consistently, transient suppression of *LpNOL* led to 16% more protoplasts viability than the control (protoplasts transformed with the *GFP*) after heat treatment (35°C) for 20 min, while suppression of *LpSGR* led to significantly decreased protoplasts viability (49% less) (Fig. 1). This result suggested that PRIDA is a valid assay to rapidly characterizing at least some heat-stress regulatory genes, such as *LpNOL* and *LpSGR*.

Yet, we shall always be cautious that the PEG4000-mediated protoplast transformation might have primed the protoplast with moderate PEG-induced osmotic stress. Therefore, parallel transformation with a gene unrelated to stress (*GFP* in this case) shall always be carried out as the control. Also, this protoplast-based 'PRIDA' protocol is likely unsuitable to characterize genes involving cell wall and/or inter-cellular stress-related pathways.

Identification of potential thermosensors, *LpTT3.1* and *LpTT3.2*, in ryegrass using 'PRIDA'

To further verify whether 'PRIDA' is suitable to test other candidate regulatory genes in heat tolerance, we cloned and characterized the ryegrass orthologous genes to rice *Thermo-tolerance 3.1* (*TT3.1*) and *TT3.2*, and named them as *LpTT3.1* and *LpTT3.2*, respectively. In rice, the *TT3.1* and *TT3.2* pair serve as potential thermosensors that over-expressing *TT3.1* or *TT3.2* led to improved or compromised heat tolerance, respectively (Zhang et al. 2022a). *LpTT3.1* and *TT3.1* share 86.4% nucleotide (nt) identity, and *LpTT3.2* and *TT3.2* share 74.4% nt identity (supplementary Fig. 1). Comparison of synonymous (*Ks*) and nonsynonymous (*Ka*) substitution rates between these orthologous genes showed that their *Ka/Ks* values were both much less than one (0.09–0.15) (supplementary Table 1), suggesting that these orthologous genes were under 'purifying selection' to keep their conserved functions as potential thermosensors across rice and ryegrass (Zhang et al. 2022a). Consistent to the *in-silico* prediction, transient over-expressing *LpTT3.1* led to significantly higher protoplast viability rates than the *GFP* control (29% more), while the contrary was true for those over-expressing *LpTT3.2* (24% less) (Fig. 2).

Taking *LpTT3.2* as the target gene, we further tested the optional temperature range (31 to 41°C) for the heat treatment on ryegrass protoplasts. The protoplasts transformed with *35S:GFP* or *35S:LpTT3.2* showed significant different viabilities when treated at 35 to 37 °C (Fig. 3). Somehow, when treated at 41 °C, the protoplasts over-expressing *LpTT3.2* adhered to each other making it impossible to count precisely. Therefore, the optional temperature range for heat treatment on ryegrass protoplasts is 35 to 37 °C. It is noteworthy that ryegrass is a cool-season grass species that is rather sensitive to heat stress (Zhang et al. 2020). Therefore, the discriminating temperature range using the 'PRIDA' system shall be optimized when applied in other plant species.

'PRIDA' is applicable to rapidly characterizing oxidative-stress regulatory genes

One side of the detrimental effect of heat stress on plants is causing the burst or accumulation of reactive oxygen species (ROS) that will aggravate cellular damage (Nadarajah 2020). As for *LpNOL* and *LpSGR* transgenic plants, their altered heat tolerance traits were both associated with stabilized or jeopardized ROS homeostasis (Yu et al. 2022; Zhang et al. 2022b). Then, we tested whether 'PRIDA' is applicable for characterizing genes regulating oxidative stress using *LpNOL* & *LpSGR* together with *LpTT3.1* & *LpTT3.2*.

H₂O₂ was added to the transformed protoplasts to final concentrations of 0, 25, 50, 75, or 100 mM, respectively. As shown in Fig. 4, both suppressing *LpSGR* and over-expressing *LpTT3.2* significantly decreased protoplast viabilities when treated with H₂O₂ at final concentrations of 50 to 75 mM; knocking-down *LpNOL* significantly increased protoplast viability when treated with 75 mM H₂O₂; while over-expressing *LpTT3.1* had no effect on protoplast viability under the oxidative stress. Notably, treatment with 100 mM H₂O₂ killed all protoplasts within 5 min, while treatment with 25 mM H₂O₂ could not discriminate the transgenes' effects (Fig. 4). In short, 50–75 mM H₂O₂ treatment is suitable for 'PRIDA' to characterize ryegrass genes against oxidative stress.

Screening E3 ligase genes involved in heat- and oxidative-stresses based on 'PRIDA'

Then, we used 'PRIDA' to screen ryegrass differentially expressed *E3 ligase* genes after heat stress (Zhang et al. 2022b; Wang et al. 2017). E3 ligases play key roles in 26S proteasome-mediated protein degradation and are indispensable in plant heat tolerance (Zhang et al. 2022a; Melo et al. 2021; Doroodian and Hua 2021). Six RING-type *E3 ligase* genes were up-regulated after short (6 hours) and long-term (21 days) of heat stress in ryegrass and were selected as candidate heat tolerance genes. As a result, over-expression of two genes (*E3-4* and *E3-12*) nearly doubled the protoplast viability after heat treatment (35 °C for 20 min), and two genes (*E3-12* and *E3-14*) improved protoplast viability upon oxidative stress (50 mM H₂O₂ for 5 min) (Fig. 5). Combining the transcriptomic data, the 'PRIDA' result narrowed our candidate genes down to three of them (*E3-4*, *E3-12*, and *E3-14*) that might play positive roles in ryegrass heat and/or oxidative tolerances.

Conclusion

The procedure of 'PRIDA' was illustrated in figure 6 with critical points pinpointed. Generation of stable transgenic ryegrass plants takes at least 7 months, and more time is needed to characterize these transgenic plants for heat and/or oxidative tolerance, while 'PRIDA' can be completed within two days. Compared with ectopic expression in yeast or in *Arabidopsis*, 'PRIDA' is an assay carried out in the native plant species (ryegrass in this case). Therefore, we believe 'PRIDA' is an optional assay to rapidly identify candidate genes in abiotic stress (e.g., heat and oxidative), and this established protocol in ryegrass can be readily adapted to other type of abiotic stresses and in other plant species.

Declarations

Authors' contributions

SL, LZ, WJ, JZ, and YC performed vector construction, ryegrass protoplast isolation and transformation experiments; SL, LZ, and BX participated in manuscript preparation; BX conceived and designed the experiments. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets supporting the conclusions and description of a complete protocol are included within the article. The pVT1629 vector is available upon request.

Consent for publication

All authors agreed to publish this manuscript.

Ethics approval and consent to participate

All authors read and approved the manuscript.

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Figures

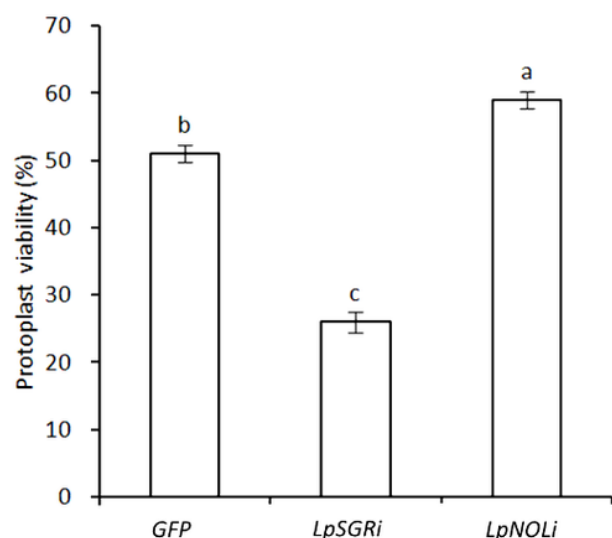


Fig. 1 Transient suppression of *LpSGR* or *LpNOL* led to suppressed or improved protoplast viabilities. The transformed ryegrass protoplasts with RNAi vectors of *LpSGR* (*LpSGRi*) or *LpNOL* (*LpNOLi*) were subjected to 35°C for 20 min. At least 30 protoplasts were counted in one scope, and data of 3 scopes were used as replicates. The data are expressed as means $\pm$ SE. Different letters above bars represent significant difference at $p=0.05$. Two independent experiments were carried out, and consistent results were obtained.

Figure 1

See image above for figure legend

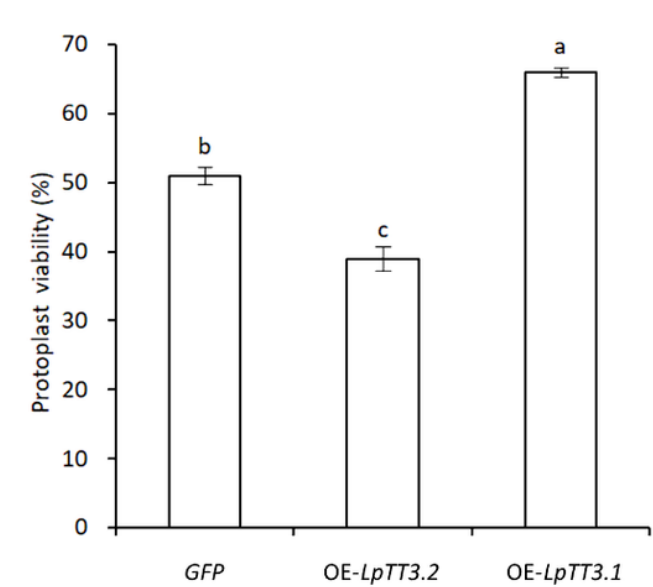


Fig. 2 Transient over-expression of *LpTT3.1* or *LpTT3.2* led to suppressed or improved protoplast viabilities. The transformed ryegrass protoplasts were subjected to 35°C for 20 min. At least 30 protoplasts were counted in one scope, and data of 3 scopes were used as replicates. The data are expressed as means $\pm$ SE. Different letters above bars represent significant difference at $p=0.05$. Two independent experiments were carried out, and consistent results were obtained.

Figure 2

See image above for figure legend

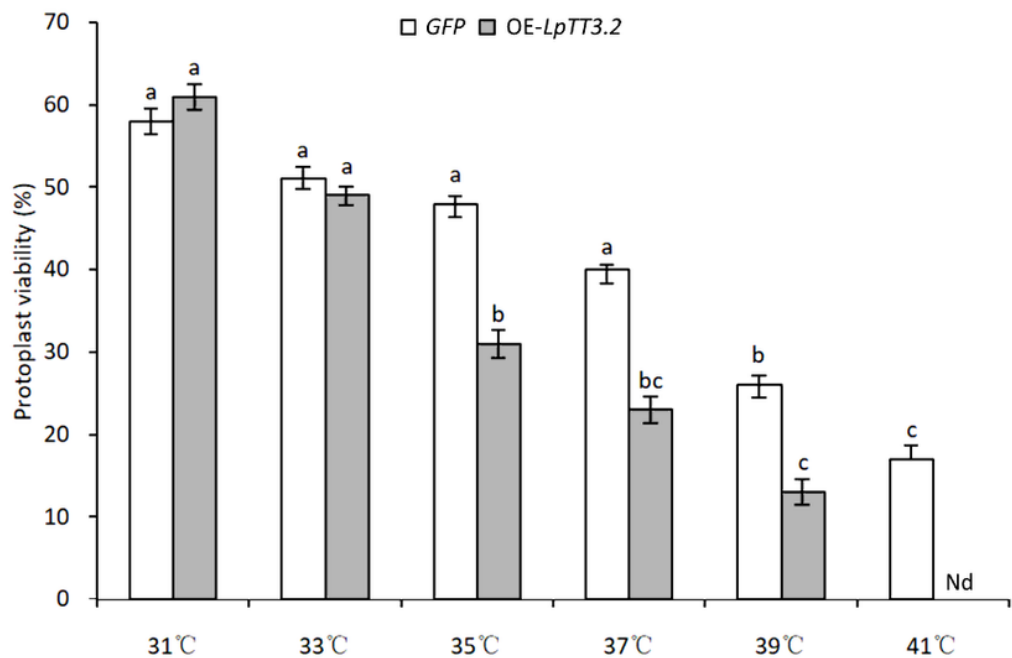


Fig. 3 Optimal temperature range for discriminating effect of *LpTT3.2* on protoplast viabilities. The transformed ryegrass protoplasts were subjected to 31-41°C for 20 min. At least 30 protoplasts were counted in one scope, and data of 3 scopes were used as replicates. The data are expressed as means $\pm$ SE. Different letters above bars represent significant difference at $p=0.05$. Two independent experiments were carried out, and consistent results were obtained.

Figure 3

See image above for figure legend

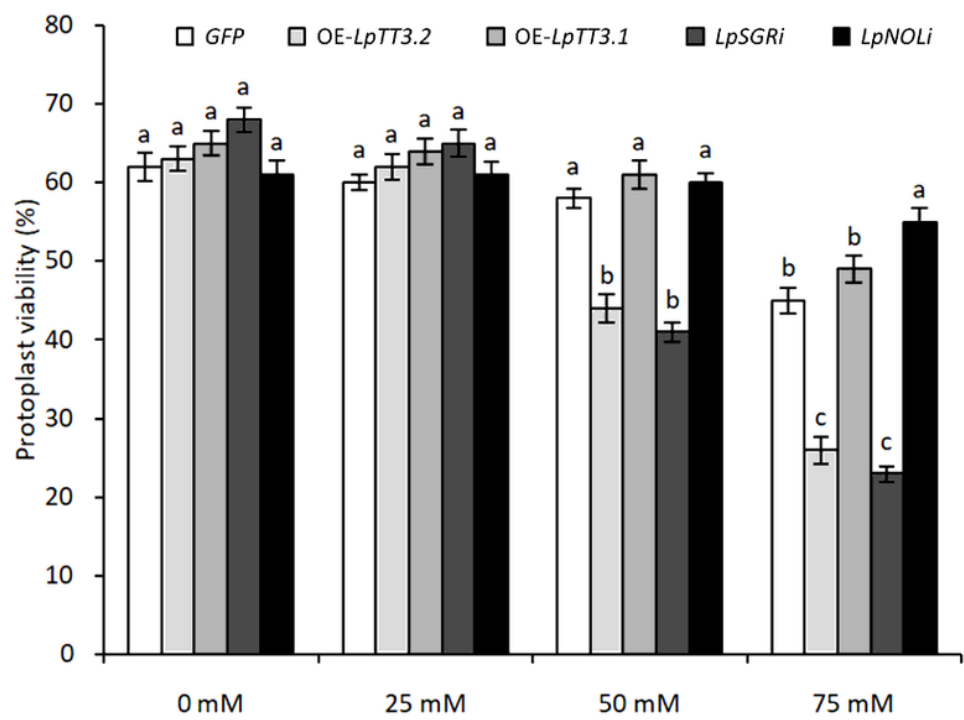


Fig. 4 Transient expression of *LpSGRi*, *LpNOLi*, *LpTT3.1*, or *LpTT3.2* led to altered protoplast viabilities subjected to H₂O₂-induced oxidative stress. At least 30 protoplasts were counted in one scope, and data of 3 scopes were used as replicates. The data are expressed as means ± SE. Different letters above bars represent significant difference at $p=0.05$. Two independent experiments were carried out, and consistent results were obtained.

Figure 4

See image above for figure legend

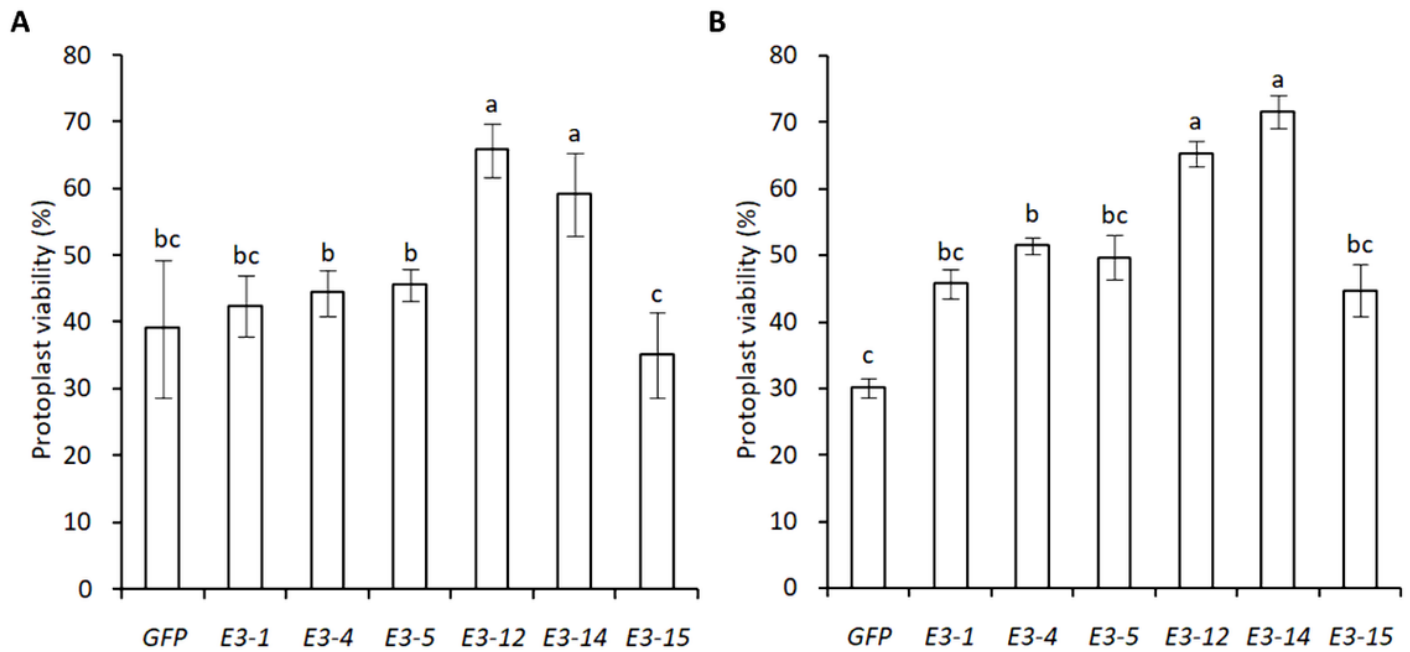


Fig. 5 Screening *E3* ligase genes involved in heat stress (A) or oxidative-stress (B) based on ‘PRIDA’. The transformed protoplasts were subjected to 35°C for 20 min (A) or to 50 mM H₂O₂ for 5 min. At least 30 protoplasts were counted in one scope, and data of 3 scopes were used as replicates. The data are expressed as means $\pm$ SE. Different letters above bars represent significant difference at $p=0.05$. Two independent experiments were carried out, and consistent results were obtained.

Figure 5

See image above for figure legend

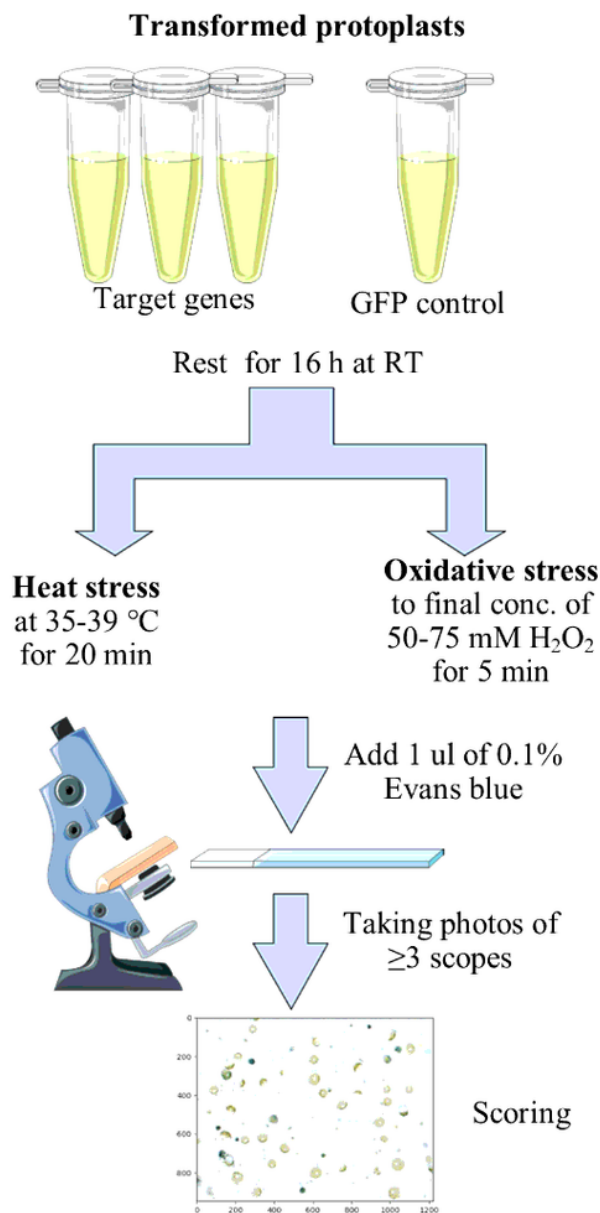


Fig. 6 Scheme of PRIDA using ryegrass mesophyll protoplasts. Noting that the parameters shall be optimized when applied in other plant species.

Figure 6

See image above for figure legend

Supplementary Files

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- [Supplementaryfile.pdf](#)