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Engineering Climate-Resilient Rice Using a Nanobiostimulant-based “Stress Training” Strategy

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Abstract

Under a changing climate, cultivating climate-resilient crops will be critical to maintaining food security. Here, we propose the application of ROS-generating nanoparticles as nanobiostimulants to trigger stress/immune responses, and subsequently increase the stress resilience of plants. We established three regimens of AgNPs-based “stress training”: seed priming (SP), leaf priming (LP), and combined seed- and leaf- priming (SLP). Trained rice seedlings were then exposed to either rice blast fungus (*M. oryzae*.) or chilling stress (10 ºC). The results show that all “stress training” regimes, particularly SLP significantly enhanced the resistance of rice against the fungal pathogen (lesion size reduced by 82% relative to un-trained control). SLP training also significantly enhanced rice tolerance to cold stress. Under cold conditions, SLP training significantly increased leaf biomass by 35% compared to controls. The mechanisms for the enhanced resilience were investigated with metabolomic and transcriptomic profiling, which show that “stress training” induced considerable metabolic and transcriptional reprogramming in rice leaves. AgNPs-boosted ROS activated stress signaling pathways by oxidative post-translational modifications of stress related kinases, hormones, and transcriptional factors (TFs). These signaling pathways subsequently modulated the expression of defense genes, including specialized metabolites (SMs) biosynthesis genes, cell membrane lipid metabolism genes, and pathogen-plant interaction genes. These AgNPs-triggered metabolic and transcriptional reprogramming enable rice plants to mount a more rapid and intense response to future stresses. This nanobiostimulant-based strategy for increasing the stress resilience of crops will increase yield vigor against a changing climate and will contribute to sustainable agriculture by reducing agrochemical use.

Main

Agricultural crops encounter a large number of stresses, and a changing climate is increasing the frequency of challenges from extreme heat, drought, flood, cold, and salinity[1]. In addition, the activity of pathogenic and pest agents such as viruses, bacteria, fungi, nematodes, and insects is also increasing under a changing climate[2]. This concurrence of abiotic and biotic stresses could result in heavy loss of agricultural production and threaten global food security[3, 4]. Notably, the current agricultural strategies for mitigating abiotic stress and biotic stress are largely siloed, inefficient, and reactive, as opposed to coordinated, efficient and prophylactic. For example, pesticides can be effective for disease control but cannot mitigate abiotic stress. Moreover, the inefficiency of pesticide delivery results in significant release into the environment and subsequent detrimental environmental and public health effects. As such, seeking sustainable strategies that simultaneously increase disease resistance and stress tolerance will be a critical tool for expanding crop productivity in a changing climate.

Importantly, plants have evolved sophisticated defense systems to combat pathogen attack and survive under marginal conditions[5]. Under such stress, plants can rapidly reprogram their transcriptome and metabolome, establishing a defense network[6]. More importantly, these transcriptional or metabolic modifications can be “remembered” by plants, resulting in a more rapid and/or stronger response upon subsequent stress exposure through “stress memory”[7, 8]. As such, plants can be “trained” to establish an

enhanced defensive capability[8]. We refer to this process as “stress training”, which involves an initial stress stimuli and subsequent plant innate defense responses. Stimuli that trigger systemic stress responses could enhance plant resistance to wide array of abiotic and biotic stresses. As such, developing such stimuli is of great importance.

Reactive oxygen species (ROS) play a crucial role in abiotic and biotic stress sensing and transduction. ROS can serve as signaling molecules, and integrate with other signaling pathways to trigger systemic defense networks[9]. Yuan *et al.* reports that ROS play important roles in the linkage between patterned triggered immunity (PTI) and effector triggered immunity (ETI)[10]. Consequently, ROS may be a stimulus that can trigger desired systemic stress response, endowing plants with enhanced resistance to multiple stressors. However, exogenous delivery of ROS to plant cells is challenging, given that ROS are highly unstable. In plants, ROS can be actively generated by the membrane-located enzyme RBOH (respiratory burst oxidase homologues) during plant-pathogen interactions[11]. Inspired by this, we hypothesize that a nanozyme that catalyzes ROS generation could serve as a nanobiostimulant to trigger wide-spectrum stress responses and enhance plant resilience.

Here, we use ROS-generating nanoparticle silver (AgNPs) as nanobiostimulant to successfully “train” rice for enhanced resistance to blast disease and cold stress. The molecular mechanisms underlying the enhanced stress were characterized by orthogonal physiological and omics endpoints. Specifically, AgNPs-generated ROS interfaced with many other signal transduction pathways to broadly activated defense genes and metabolites, increasing resistance to subsequent abiotic and biotic stresses. These results demonstrate that nano-based “stress training” could safely and sustainably enable engineer of climate-resilient crops, proving to be an effective strategy to increase crop production and combat food insecurity.

“Stress Training” Enhanced Resilience of Rice to Biotic and Abiotic Stresses

Blast disease caused by the fungal pathogen *Magnaporthe oryzae* is the most serious disease of rice[12]. As such, *M. oryzae* was selected as the biotic stress for AgNPs-based “stress training” experiments. Three types of “stress training” were established, including seed-priming (SP) (seed treatment with 40 mg/L AgNPs for 24 or 48 h), leaf-priming (LP) (foliar spray of 40 mg/L AgNPs to rice seedlings at 1, 3, 5, and 7 d before stress exposure), and combined seed- and leaf- priming (SLP), resulting in 15 treatments (Fig. 1A). LP training was initiated 30 days after germination. At day 37, primed rice leaves were detached and inoculated with *M. oryzae*. At 7 days post inoculation, lesions were evident in all rice leaves (Fig. 1B). However, lesion size in leaves pre-trained with AgNPs were significantly smaller (1.9 ~ 5.8 mm) than that in un-trained leaves (11.5 mm) (Fig. 1C), indicating the enhanced disease resistance. Notably, SLP yielded greater resistance to *M. oryzae* than SP or LP (Fig. 1C). Specifically, SLP-24-7d and SLP-48-7d are comparable, exhibiting the greatest disease resistance among all the treatment (reduction of lesion size by 82% and 83%, respectively, relative to control). Importantly, foliar spray of AgNPs 7 days before inoculation resulted in the best resistance, followed by 5 d, 3 d, and 1 d, indicating a time dependence of resistance onset after foliar application but prior to fungal attack.

To confirm the disease suppression ability of “stress training”, an *in Vivo* plant assay was conducted where trained 37-d-old rice seedlings (Control, SP-24, LP-7 d, and SLP-24-7d) were treated with the *M. oryzae*. Seven days post inoculation, disease symptoms (blast lesions) were observed in all rice leaves, although again it was clear that blast disease in leaves pre-trained with AgNPs was less severe than in controls (**Figure S1A**). As shown in **Figure S1B**, SLP training reduced leaf blast severity from 6 to 3, indicating substantial disease resistance enhancement. These results are in consistent with the *in Vitro* detached leaf assay, highlighting the disease suppression ability of “stress training”, especially SLP.

The ability of “stress training” on rice cold tolerance was investigated as well. Trained 37-d-old rice seedlings (Control, SP-24, LP-7d, and SLP-24-7d) were exposed to low temperature (10 ºC) growth conditions for 10 days. At day 2, growth inhibition was evident in the control and SP-24 seedlings. Whereas trained seedlings, particularly SLP-24-7d, were noticeably larger and greener (**Figure S2A**); this pattern lasted until day 10 (**Figure S2B**). At the end of the exposure, the leaf biomass of LP-7d and SLP-24-7d were significantly ($p < 0.05$) greater (16.6% and 34.6%, respectively) than controls (**Figure S2C**), demonstrating the efficacy of “stress training” against cold stress. Interestingly, SLP-24-7d yield the greatest disease resistance and cold tolerance. We hypothesize that AgNPs-generated ROS, as signaling molecules, triggered defense responses, resulting in the activation of systemic acquired resistance (SAR) and systemic acquired acclimation (SAA) that simultaneously enhanced biotic and abiotic stress resistance.

Metabolic Reprogramming In Rice Leaves

Metabolomics and transcriptomics analyses were employed to elucidate the molecular nature of defense network in trained rice leaves. As metabolites are the final products of biological processes, we first examined the metabolome of leaf (37-d-old, post priming and before inoculation) under different “stress training” regimes (control, SP, LP, SLP). Through GC-MS-based metabolomics, a total of 275 metabolites were identified and semi-quantified. A sparse partial least squares-discriminant analysis (sPLS-DA) model was used to assess metabolome variation between groups. The score plot of sPLS-DA shows a clear separation between the four groups (Fig. 2A), suggesting that “stress training” (SP, LP, and SLP) caused global metabolic changes rice leaves. Notably, the leaf-priming groups (LP and SLP) clearly separate from the non-leaf-priming groups (control and SP) along component 1 (PC1); while hydro-priming groups (control and LP) separate with AgNPs-priming groups (SP and SLP) along PC2. This indicates that leaf-priming and seed-priming induced different patterns of metabolic changes in the leaves. Figure 2A also demonstrates that SLP induced the most pronounced metabolic changes as compared to SP and LP. This is verified by univariate *t*-test analysis (see Venn diagram in Fig. 2B); the number of differentially accumulated metabolites (DAM) induced by SLP, LP, and SP is 187, 181, and 118, respectively. As described above, SLP training generated the greatest resistant to *M. oryzae* and tolerance to chilling stress. Therefore, we speculate that the observed modulated metabolite profile directly correlates with enhanced resistance.

Variable importance in projection (VIP) scores of PC1 (control and SP *vs.* LP and SLP) were calculated to identify the metabolites contributing to the enhanced resilience. Interestingly, the top 30 metabolites show a very similar pattern across the different groups, being less abundant in leaf-priming groups (LP and SLP), and more abundant in non-leaf-priming groups (control and SP) (Fig. 2C), indicating that leaf-based "stress training" (SP and SLP) induced systemic shifts in rice metabolome. Notably, there is a systemic down-regulation of amino acids (beta-alanine, homoserine, isothreonic acid, asparagine, aspartic acid, glutamic acid, glutamine, isoleucine, lysine, methionine, proline, lysine, threonine, glycine, serine, tryptophan, tyrosine, phenylalanine, citrulline, and ornithine) in LP and SLP groups, as compared to control and SP group (**Figure S3**). Amino acids are building blocks for protein biosynthesis and serve as precursors for a diverse set of plant specialized metabolites (PSM)[13]. For instance, aromatic amino acids (tryptophan, phenylalanine, and tyrosine) serve as precursors of PSM involved in defense networks, such as salicylic acid, flavonoids, alkaloids, tocopherols, auxins, and cell wall component lignin[14]. In addition, methionine is a precursor of phytohormone ethylene[15]; lysine is the precursor of defense signaling molecular pipecolic acid. Thus, the systemic decrease of free amino acids in LP and SLP trained leaves may possibly due to the need for synthesis of defensive proteins and (or) specialized metabolites in response to AgNPs-generated ROS.

In addition to amino acids, several sugars (fructose-1,6-bisphosphate, ribulose 5-phosphate, glucose-6-phosphate, glycerol 3-phosphate, trehalose-6-phosphate, xylose, galactose, melibiose), nucleotides (cytidine-5-monophosphate, guanine, uracil, xanthine), and fatty acids (arachidic acid, behenic acid, beta-hydroxymyristic acid, d-erythro-sphingosine) were systemically reduced in leaf-priming groups (LP and SLP) compared to non-leaf-priming groups (control and SP) (**Figure S3**). These primary metabolites (sugars, nucleotides, and fatty acids) are the building blocks of many important macromolecules, including RNA, DNA, and lipids. The decrease of these metabolites may suggest that there is considerable shift in carbon flow from primary metabolites to secondary metabolites, resulting in the production of defense proteins, metabolites, and the cell-wall component lignin, all in response to AgNPs-generated ROS during "stress training"[14].

Additionally, several TCA cycle intermediates were significantly increased (citric acid, aconitic acid, alpha-ketoglutarate, succinic acid, malic acid) or decreased (isocitric acid and fumaric acid) in leaf-priming groups (SLP and LP) relative to non-leaf-priming groups (**Figure S3**). This boosting of the TCA cycle upon leaf-priming may also promote defense protein synthesis. TCA cycle acceleration can stimulate protein synthesis by two mechanisms: 1) providing essential precursors for ATP production, 2) by providing amino acids required for protein synthesis. For instance, glutamate, alanine, and aspartate are intermediates of glycolysis and TCA cycle[16]. The TCA cycle also releases metabolites into the cytosol, where they serve as building blocks for the synthesis of other macromolecules synthesis, e.g., lipid and nucleotides[17]. Therefore, TCA cycle enhancement may directly lead to the greater biosynthesis of defense related macromolecules, such as amino acids, lipid, and nucleotides.

In addition to providing precursors for macromolecule biosynthesis, the TCA cycle is also directly involved in biotic stress response and cellular redox homeostasis. For instance, alpha-ketoglutarate (α-KG) has been

reported to have a role in the activation and regulation of macrophage immunity[18]. In addition, α-KG acts as an antioxidant agent that directly reacts with H_2O_2 during the formation of succinate, water, and CO_2[19]. Interestingly, the level of α-KG and its derived metabolite, L-2-hydroxyglutaric acid (LGA), were significantly enriched in LP and SLP trained leaves, and these biomolecules have a strong positive relationship with rice blast resistance (Pearson's correlation coefficient $R^2 = 0.9$). Given this, it is likely that α-KG plays an important role in activating the rice leaf defense network.

Metabolites are the downstream products of metabolic processes, and as such, these biomolecules can also actively modulate biological process and phenotype[20]. Plants have evolved dynamic metabolic pathways that produce a range of structurally and functionally diverse specialized metabolites (terpenes, flavonoids, alkaloids) that assist in biotic and abiotic stress tolerance[21]. Our data show that a number of phenylpropanoid derivatives, including benzoic acid, cis-caffeic acid, hydrocinnamic acid, isoferulic acid, quinic acid, were significantly up-regulated in leaf-primed groups compared to non-leaf-primed plants (**Figure S3**). In addition, shikimic acid and phenylalanine, precursors of these phenolic compounds, were also up-regulated in leaf-primed groups. Phenolics are important metabolites which protect plants from ROS attack. It appears that AgNPs-generated ROS triggered defense responses in leaves, and subsequently, shikimate and phenylpropanoid pathways were activated in response to the ROS burst to produce protective metabolites such as phenolics. In addition to phenolics, ascorbic acid, another important intracellular ROS scavenger (redox regulators), was significantly increased in leaf-primed groups compared to non-leaf-primed groups (**Figure S3**). Collectively, the upregulation of these antioxidant metabolites likely contributes to the enhanced resistance of rice leaves to subsequent stressors, such as pathogen attack and chilling stress, by boosting antioxidant defense systems.

In addition to acting as ROS-scavengers, some specialized metabolites can act as signals for reprogram gene expression[22]. We found that salicylic acid (SA), an important phytohormone, was significantly increased in trained rice leaves compared to controls (**Figure S3**). SA is an immunity-related phytohormone that is synthesized after pathogen infection to induce systemic acquired resistance (SAR)[23]. SA is also involved in activation of abiotic stress responses[24]. As such, SA upregulation may contribute to the observed enhanced resistance to rice blast and chilling stress. Similar to SA, pipecolic acid (Pip) is an immune-regulatory plant metabolite that plays important roles in establishment of plant SAR and basal immunity. The level of Pip was also significantly increased in trained rice leaves (**Figure S3**). Bernsdorff *et al.*[25] reported that Pip can act as signaling molecules to activate SAR and defense priming and can have significant crosstalk with SA. A recent report shows that exogenous application of N-hydroxypipecolic acid triggered SAR and enhanced the resistance of wheat (*Triticum aestivum*) to the fungal pathogen *Fusarium graminearum*[26]. Together, the up-regulation of SA and Pip are a strong indicator that AgNPs-generated ROS triggered systemic acquired resistance, which may then provide resistance to broad-spectrum of pathogens.

Collectively, the metabolomics data reveal a holistic view of metabolic changes in leaves with different types of "stress training" (Fig. 3). During leaf-priming, AgNPs-generated ROS reprogrammed nitrogen and

carbon metabolism and activated specialized (secondary) metabolite pathways to boost production of antioxidants and signaling metabolites. These metabolic responses facilitated rice to preparation for subsequent abiotic and biotic stresses. Notably, seed-priming (SP) also resulted in unique metabolic changes in rice leaves that contribute to enhanced stress resistance (more discussion regarding SP induced metabolic changes are in the **SI**). Consequently, combined seed- and leaf priming provides maximum stress resistance to treated rice.

Transcriptomic Reprogramming In Rice Leaves

Metabolite concentrations are modulated by transcription, although metabolites may also interact with transcription factors to regulate gene expression networks[27]. RNA-seq-based transcriptome profiling was performed to provide a holistic view of the changed molecular networks and to better understand the mechanisms by which “stress training” enhanced stress resistance. Principal component analysis (PCA) reveals that all the “stress training” groups clearly separate from control along PC1 (LP) and PC2 (SP), as well as from SLP (Fig. 4A), indicating that all the “stress training” triggered global transcriptional reprogramming in rice leaves. An analysis of differentially expressed genes (DEGs) ($q < 0.05$; foldchange > 2 or foldchange < 0.5) shows that SLP yielded most transcriptional changes (2049 DEGs), followed by LP (1891 DEGs) and SP (409 DEGs) (Venn diagrams in Fig. 4B). This pattern of response is similar to the metabolomics data (Venn diagrams in Fig. 2B), indicating that SLP training induced most pronounced molecular reprogramming in rice leaves.

SLP resulted in the upregulation of 783 genes, and downregulation of 1266 genes (**Figure S4**). Gene Ontology (GO) analysis of these DEGs reveals a striking enrichment in categories associated with defense and general stimuli responses, cellular homeostasis, secondary metabolic process, lipid and carbohydrate metabolism, protein modification, and cell death (Fig. 4C). This indicates that SLP “stress training” triggered a rather dramatic defense response in rice leaves. KEGG pathway analyses (SLP *vs.* Control, top 20) reveals that some DEGs are highly enriched in plant hormone signal transduction (salicylic acid-, ethylene-, gibberellin-, and auxin- related) and MAPK signaling pathways (Fig. 4D). Activation of MAPKs is a critical early cellular event of plant immune responses[28]. These results clearly demonstrate that AgNPs-generated ROS may activate local and systemic defenses in rice leaves.

Unlike hormones that are receptor specific, ROS as signaling molecules do not have specific receptors. Whereas ROS are able to reversibly oxidize redox-sensitive cysteine residues on target proteins[29]. Through oxidative post-translational modifications (oxi-PTMs), ROS can alter the conformation and function of multiple cytoplasmic proteins [30]. ROS-mediated redox-driven oxi-PTMs can regulate the activity of different kinases, phosphatases, transcription factors (TFs)[30]. We report that a number of kinases were either activated or inhibited in SLP trained rice leaves, including leucine-rich repeat-containing protein kinase, MAP 3-kinase (MAP3K), lectin-like protein kinase, OsWAK receptor-like protein kinase, tyrosine protein kinase, calcium/calmodulin dependent protein kinases (CAMK), and cysteine-rich receptor-like kinases (CRKs) (**Figure S5A**). Collectively, these kinases play important roles in immune response. For

instance, the receptor-like kinase (RLK) gene-family plays a central role in signaling during pathogen recognition, and the subsequent activation of plant defense mechanisms[31]. Lectin RLKs are involved in the perception of external abiotic or biotic stimuli, such as pathogen attack or changes in temperature[32, 33]. Under salt stress, RLK can sense cell wall defect or damage signals so as to maintain cell wall integrity[34]. Conversely, down-regulation of protein phosphatases and tyrosine phosphatase were evident in AgNPs-primed leaves (**Figure S5A**). The inhibited gene expression of phosphatases is possibly due to ROS induced oxidation. Tyrosine phosphatase contains a catalytic cysteine residue that loses enzyme function when oxidized and has been shown to undergo diverse modes of oxidative PTMs[35].

The metabolomics data demonstrated that the hormone salicylic acid was significantly over-produced in SLP trained leaves. Previous studies have shown extensive crosstalk between salicylic acid and ethylene (ET) signaling pathways[36]. We found that ethylene-insensitive protein 3 (EIN3) (2.5-fold) and ethylene-responsive TF (2-3.8-fold) were highly expressed in SLP trained leaves (**Figure S5B**). SA and ET response pathways serve as the backbone of the induced defense signaling network, with other hormone response pathways feeding into it, including auxin, gibberellins, cytokinins, and brassinosteroids[36]. Gibberellin-receptor, gibberellin 2-beta-dioxygenase, and auxin-responsive SAUR gene, were increased 2 ~ 6-fold (**Figure S5B**). These hormone-regulated defense signaling plays vital roles in plant resistance to pathogens and tolerance to drought, salinity, heat, cold and flooding[37]. These data clearly demonstrate that “stress training” triggered activation of the hormone-mediated defense network.

TFs are downstream of cellular signaling pathways, and also subject to redox control through a number of mechanisms[35]. We report that SLP altered the expression of several stress-related TFs in rice leaves, including the WRKY, MYB, bZIP, AP2, TGA1, NAC, zinc finger, homeobox associated leucine zipper, and ethylene-responsive TF (**Figure S5C**). These are redox-regulated TFs that are known to play important roles in response to pathogens and abiotic stress[38]. Among them, WRKY and bZIP (basic leucine zipper) are involved in the SA-dependent activation of PR genes. WRKY are one of the TFs families that play important roles in immune responses[39]. For example, WRKY has been reported to negatively regulate the basal resistance of *A. thaliana* against pathogenic *Pseudomonas syringae*[40]. In addition, bZIP has been shown to have involvement in responses to abiotic stresses such as drought, salinity, and cold by regulating the expression of stress-responsive genes[41]. Fichman *et al.* reported that MYB30 is a key regulator that links systemic ROS signaling with systemic acquired acclimation (SAA) in *Arabidopsis thaliana*[42]. In summary, SLP training triggered multiple stress signaling pathways, that collectively formed an interconnected stress regulatory network.

TFs also modulate the expression of defense genes. As such, we sought to identify defense genes which may contribute to the enhanced chilling tolerance and disease resistance. As discussed above, the metabolomics data indicate that a number of phenolic compounds were upregulated upon SLP treatment. Interestingly, KEGG pathway analysis reveals that the flavonoid and phenylpropanoid biosynthesis pathways, both of which are involved in phenolics production, were enriched in rice leaves upon SLP treatment (Fig. 4D). These data indicate that flavonoids biosynthesis was activated upon SLP training.

Flavonoids have been reported to protect plants against a variety of stresses, including high temperature, cold, drought, and pathogen attack,[43] either as antioxidants or signalling molecules. The upregulation of these secondary metabolites and their genes likely play important roles in enhanced stress resistance. Additionally, a number of pathways involved in producing a range of specialized metabolites were upregulated upon SLP training, including the biosynthetic pathways for monoterpenoid, benzoxazinoid, betalain, zeatin, and brassinosteroid (Fig. 4D). Metabolites produced from these biological pathways have known involvement in protecting plants against abiotic and biotic stresses[44]. For instance, betalains can protect plants from stress damage by acting as ROS scavengers and osmotic substances[44]. Zeatin is an important hormone in plant biotic and abiotic stress response. Benzoxazinoids are defense compounds against pathogens attack,[45] and brassinosteroids are plant hormones that protect the plants from biotic and abiotic stresses[46]. It is noteworthy that a key gene family involved in the biosynthetic pathways of many of these specialized metabolites are Cytochrome P450 monooxygenases (P450s), which were dramatically up-regulated in SLP trained leaves (**Figure S5D**). P450s regulate and catalyze the biosynthesis of defense-related specialized metabolites, including flavonoids, phytohormones, tropane alkaloids, cutin, and cuticular wax[47]. Collectively, transcriptome profiling reveals that leaf-priming, particularly SLP, activated a range of specialized metabolic pathways that are collectively responsible for the enhanced resistance to rice blast fungus and cold stress.

In addition to specialized metabolites biosynthesis, leaf-priming induced other transcriptional changes that likely contributed to enhanced resistance. The KEGG analysis reveals that fatty acid elongation, glycerolipid metabolism and glycerophospholipid metabolism were upregulated upon SLP “stress training” (Fig. 4D). These lipid metabolism-related pathways have been shown to play important roles in plant defense against a range of abiotic and biotic stresses. Glycerol-3-phosphate acyltransferase (GPAT), a key gene in glycerophospholipid metabolism, was increased 3 ~ 8-fold upon SLP training (**Figure S5D**). GPAT gene is associated with fatty acid unsaturation and plays a pivotal role in cold resistance in a number of plant species[48, 49]. Plant 3-ketoacyl-CoA synthase (KCS), which is involved in the biosynthesis of very long chain fatty acids (VLCFAs)[50], was increased 20-fold upon SLP stress training (**Figure S5D**). VLCFAs play important roles in biosynthesis of cuticular waxes, a hydrophobic film deposited on aerial tissue surfaces and provides protection against pathogen infection[51]. In addition, lipid-associated plant defense responses are largely facilitated by the activation of lipases (lipid hydrolyzing proteins), which cleave or transform lipid substrates in various subcellular compartments[52]. We found that lipases were highly expressed in rice leaves with SLP training (**Figure S5D**). Cellulose is the main component of plant cell walls and is synthesized by plasma membrane-localized cellulose synthases[53]. In rice leaves with SLP training, the expression of cellulose synthases-like protein increased 23-fold compared to controls (**Figure S5D**). In addition, extracellular hydroxyproline-rich glycoprotein (HRGP), which contributes cell wall fortification, was also highly expressed in SLP trained leaves (**Figure S5D**). These data indicate that cell wall reinforcement occurred in response to AgNPs-generated ROS. The immune response to pathogen invasion involves the modification of membrane structure and the release of membrane lipid molecules through hydrolysis[52]. Cold stress affects plant cell membrane fluidity and increasing membrane stability is known

to help plant to acclimate to low temperatures. As such, the changes in lipid metabolism-related genes noted above most likely contributed to enhanced resistance to rice blast and to tolerance to cold stress.

KEGG pathway analysis also show that starch and sucrose metabolism, as well as nitrogen metabolism, were down-regulated upon SLP training (Fig. 4D). This supports our hypothesis that “stress training” would shift carbon fluxes from growth-related primary metabolism to defense metabolism, including biosynthesis of a diverse set of defense-related secondary metabolites and phytohormones. This transcriptomics analysis (Fig. 5) significant expands our understanding of the molecular mechanisms underlying enhanced plant defense.

Conclusion And Future Perspective

Here, we propose a straightforward “stress training” strategy to enhance plant resistance against biotic and abiotic stresses. During the training, ROS-boosting AgNPs can trigger defense responses, resulting in significant metabolic and transcriptomic reprogramming that enables a rapid and intense response to subsequent stresses. In this approach, AgNPs can be regarded as a “plant vaccine”. Vaccination at the seed germination stage (SP) and seedling stage (LP) both increase the resistance, although combined SLP provides nearly additive resistance. This nanobiostimulant-based “stress training” is a preventive approach to protect plants against pathogen attack or adverse conditions and can be a highly effective strategy to engineer climate-resilient crops. For leaf-based “stress training”, priming time is a critical factor that drives resistance to rice blast fungus and cold stress. As such, weather and pest/pathogen activity forecasts can be integrated into this “stress training” approach. With accurate knowledge of the coming stress, precision training regimes can be established to achieve the optimal benefit. The Chinese description of this strategy is to “shoot the arrow when there is target” ( ). Importantly, with stress training-induced SAR and SAA, rice should have broad-spectrum disease resistance and multi-stressor tolerance. Future studies can evaluate the efficacy this stress training strategy on other diseases and stressors, such as rice sheath blight and salt or heat tolerance, as well as for other crops species. It is possible that there is growth-defense trade-off, and life cycle studies will need to thoroughly and quantitative evaluate this balance, including impacts on yield and grain quality. Collectively, this strategy of stress vaccination has great potential to significantly contribute to sustainable agriculture by engineering climate-resilient crops that require decreased agrochemical while simultaneously generating greater yield.

Materials And Methods

AgNPs Characterization

The AgNPs was purchased from Pantian Nanomaterials Co., LTD. (Shanghai, China). The AgNPs are spherical in shape, as characterized by transmission electron microscopy (TEM). The hydrodynamic diameter and ζ potential of the AgNPs suspension (40 mg/L) are 160 ± 8.7 nm and − 20.2 ± 0.4 mV as measured by dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern). AgNPs have peroxidase-like

catalytic activity; electron spin resonance (ESR) spectra show that AgNPs can induce ·OH in the presence of H_2O_2.

Experimental Design- “stress Training”

We designed three regimes of “stress training”: seed-priming (SP), leaf-priming (LP), and combined seed- and leaf- priming (SLP), resulting in 15 treatments (**Figure S1**). For SP, rice seeds were soaked in 40 mg/L AgNPs for 24 or 48 h, followed by planting in pots containing potting soil in greenhouse for 30-d of cultivation. For LP, seeds were primed with DI water, and cultivated for 30-d in a greenhouse. The 30-day-old rice seedlings were then foliar spray with 40 mg/L AgNPs (approximately 5 mL/plant) at 1, 3, 5, and 7 d before stress exposure. For SLP, seeds were first primed with AgNPs for 24 or 48 h, and then foliar sprayed at 30-d. At day 37, the rice “trained” seedlings were exposed to biotic (rice blast fungus) or abiotic stress (cold stress). Assays of fungus inoculation are shown below. In case of cold stress exposure, 37-day-old untrained (control) or trained (SP, LP, and SLP) rice seedlings were transferred to an artificial climate chamber (10 ºC) with a 14 h light/10 h dark photoperiod, with approximately 60% humidity. During 10 days cold treatment, rice seedlings were photographed every two days.

Disease Resistance Assays

To evaluate the performance of AgNPs-based “stress training” on blast resistance, leaves were detached from 37-day-old rice seedlings and inoculated with *M. oryzae*. The blast fungus *Magnaporthe oryzae* was grown on an oat tomato agar plate at 25 ºC in dark for 7 days and the surface of the fungal growth was scraped with a toothbrush. The scraped plates were cultivated at 25 ºC for 3 days to induce sporulation. The conidia were then suspended at $1*10^5$ spores/mL in a 6-Benzylaminopurine (6-BA) solution. Three µL of conidia was inoculated onto wounded rice leaves detached from 37-d old rice seedlings. Inoculated rice leaves were incubated in a growth chamber at 26 ºC with 90% humidity and in the dark for the first 24 h, followed by a 12 h/12 h light/dark cycle for 7 days. The disease severity was assessed at 7 d after inoculation. Rice blast resistance was evaluated by measuring the size of the disease lesions on leaves using Image J.

For spray inoculation, trained (SP, LP, SLP) 37-day-old rice seedlings were sprayed with spore suspensions ($5*10^5$ spores/mL) at 5 mL per plant. After spray inoculation, rice seedlings were kept in darkness at 28 ºC for 24 h, followed by cultivation in growth chamber for 7 days at 12 h/12 h (day/night) and 90% relative humidity. Lesion on the leaves of intact plants were scored from 0 (resistant) to 6 (susceptible) according to a standard evaluation method[57].

Metabolite Profiling In Rice Leaves

Non-targeted metabolite profiling of rice leaves was performed via gas chromatography–mass spectrometry (GC-MS). Rice leaves sampled from 37-day-old trained (SP, LP, SLP) or untrained (control) rice

seedlings were flash-frozen in liquid nitrogen for metabolic quenching. Frozen tissues were ground to fine power in liquid nitrogen using a mortar and pestle and stored at -80 ºC until extraction. The metabolites in the leaf powder were extracted using 80% cooled methanol amended with 2-chloro-l-phenylalanine as internal standard. The extracted compounds were derivatized using methoxylamine hydrochloride and N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA). The derivatized samples were analyzed with an Agilent 7890B gas chromatography system coupled to an Agilent 5977A mass-selective detector (Agilent Technologies Inc., Santa Clara, CA). The column employed was a DB-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 μm; Agilent J&W Scientific, Folsom, CA, USA). Quantification was reported as peak height using unique ion as default. Metabolites were unambiguously assigned by BinBase identifier numbers using retention index and mass spectrum as the two most important identification criteria. More details regarding sample derivatization and GC-MS analysis are referenced in previous study[58].

For metabolomics data analysis, a supervised partial least-squares discriminant analysis (sPLS-DA) clustering method was run on the dataset using MetaboAnalyst 5.0 (http://www.metaboanalyst.ca/).[59] Before sPLS-DA, data normalization (normalization by sum) was performed for general-purpose adjustment based on the differences among samples, and data transformation (log transformation) was conducted to make individual features more comparable. The Variable Importance in Projection (VIP) is the weighted sum of the squares of the sPLS-DA analysis, and indicates the importance of a variable to the model.[60] A variable with a VIP greater than 0.1 was recognized as responsible for separation, and was defined as a discriminating metabolite.[61]

Transcriptomics Profiling In Rice Leaves

For RNA-seq analysis, rice leaves collected from 37-day-old trained (SP, LP, SLP) or untrained (control) plants were thoroughly washed and flash-frozen in liquid nitrogen, then ground into fine powder using pestle and motor. Total RNA from rice leaves was extracted using the TRIzol Regent (Invitrogen CA, USA) according to the manufacturer's instructions. The quality of the extracted RNA samples was evaluated using Agilent 2100 Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). RNA-seq libraries were prepared using VAHTS Universal V6 RNA-seq Library Prep Kit. The transcriptome sequencing and analysis were conducted by OE Biotech Co., Ltd (Shanghai, China) using the Illumina HiSeq xten/NovaSeq 6000 sequencer (Illumina). Four biological replicates for each treatment were sequenced. Differentially expressed genes (DEGs) were screening out by DESeq2[62], with a Q value < 0.05 & fold change > 2 or foldchange < 0.5 as the threshold value. Based on the hypergeometric distribution, gene ontology (GO) and KEGG pathway analysis of the DEGs were determined to screen the significant enriched term. The column, chord and bubble diagrams of the significant enrichment term were drawn with R (v 3.2.0).

Single Particle (Sp) Icp-ms

SP-ICP-MS was used to investigate particle size and concentration of silver particles or ions in rice leaves after foliar spraying with AgNPs for 2 days. Before SP-ICP-MS analysis, rice leaves were digested with

Macerozyme R-10 enzyme. After digestion, an Agilent 8900 ICP-MS (Santa Clara, CA, U.S.) equipped with concentric nebulizer, conical spray chamber, skimmer cones, and quartz torch was used to perform SP-ICP-MS analysis. The SP-ICP-MS method setup, data collection, and analyses were conducted with the Single Nanoparticle Application Module (method wizard) in the Agilent ICP-MS MassHunter software (Version C.01.03 Build 505.16 Patch 3).

Agnps Subcellular Distribution Within Rice Leaves

The presence and localization of AgNPs in rice leaves were assessed by transmission electron microscopy (TEM) according to Li et al.[63] Two days after foliar spray of AgNPs, the leaf was cut into approximately 0.5 mm*3 mm pieces. The samples were fixed with 2.5% glutaraldehyde, dehydrated with ethanol, and transferred into Spurr's resin for embedding. The resin-embedded samples were cut into 80-nm-thin cross-sectioned films using a Leica EM UC7 Ultramicrotome and were imaged by TEM (Hitachi H-7650). The particle size distribution in TEM micrographs was evaluated using Nano Measurer 1.2.

Ros Measurement

ROS levels were quantified by bioreader using 2'-7'dichlorofluorescin diacetate (DCFH-DA) as fluorescent probe according to Huang et al.[64] with modification. The cleaned leaves were ground into a fine powder with liquid nitrogen. One mL Tris-HCl (10 mmol/L, pH = 7.2) was added to 80 mg of sample. After centrifugation at 1000 g for 30 min, 800 µL Tris-HCl and 100 µL DCFH-DA (10 µmol/L) were added to 100 µL supernatant. Fluorescence intensity of DCFH-DC (488 nm excitation, 525 nm emission) was measured using a microplate reader (Synergy H4 Hybrid Reader, BioTek, America).

Statistical Analysis

Except metabolomics and transcriptomics data, mean values for each measured parameter were compared using one-way analysis of variance from SPSS (version 26, IBM) or one-tailed, two-sample Student's t-tests from Microsoft Excel. At a significance level of $p \leq 0.05$ is regarded as significant. Figures were generated with SigmaPlot 14.0 (Systat Inc., Richmond, USA).

Supporting Information. Materials and methods: Assessment of antifungal activity of AgNPs, determination of chlorophyll, MDA, total phenol content and total antioxidant capacity in rice leaves. Results and discussion: Changes of chlorophyll content in rice leaves before stress (7 days after leaf priming), Physiological and biochemical changes of rice after cold stress, Metabolic reprogramming in rice leaves after "stress training" (seed priming). Disease lesions on rice leaves from an *in Vitro* assay (**Figure S1**). Growth of rice seedlings exposed to low temperature (10ºC) (**Figure S2A, B and C**). Heatmap of altered metabolites (**Figure S3**). The number of differentially expressed genes under SLP "stress training" (**Figure S4**). Heatmap of differentially expressed genes in rice leaves under SLP "stress training" (**Figure S5**). Heatmap of differentially expressed genes in rice leaves under SLP "stress training" (**Figure S6**). ROS level in rice leaves at day 1, day 3, day 5 and day 7 after spraying AgNPs and the EPR results showing the

generation of hydroxyl radicals (**Figure S7A and B**). MDA content in rice leaves 7 days post AgNPs spray (**Figure S8**). The spore germination rate and the conidia morphology with or without AgNPs (**Figure S9A and B**). Transmission electron microscopy (TEM) images of AgNPs (**Figure S10**). Chlorophyll content of rice leaves at day 7 after leaf priming (**Figure S11**). Physiological and biochemical changes of rice after cold stress (**Figure S12A, B, C and D**). Metabolite profile changes in seed primed rice leaves (**Figure S13**). This material is available as Supporting Information.

Declarations

ACKNOWLEDGMENTS

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Tables

Table 1 Mineral nutrients content in rice leaves (mg/kg dry weight).

	K		Ca		Fe		Zn	
Control	31235 ± 559		5398 ± 75		145 ± 10.0		76.9 ± 4.0	
SP	31697 ± 939		5034 ± 143	**	178 ± 9.5	*	119.4 ± 31.6	*
LP	34627 ± 809	***	5595 ± 108	*	222 ± 25.7	*	91.5 ± 9.3	*
SLP	35687 ± 324	***	5912 ± 182	**	197 ± 16.6	*	101.2 ± 10.5	**
	Na		**Mg**		**Mo**		**Cu**	
Control	370 ± 153		2524 ± 42		2.6 ± 0.9		9.9 ± 0.3	
SP	112 ± 24	*	2462 ± 67		1.2 ± 0.2	*	11.1 ± 0.6	*
LP	126 ± 54	*	2474 ± 32		1.2 ± 0.3	*	7.8 ± 0.4	***
SLP	15 ± 6	**	2304 ± 42	***	0.8 ± 0.4	*	6.4 ± 1.1	***

Leaves were taken from 37-day-old untrained (control) or trained (SP, LP, SLP) rice seedlings. SP: seed priming with AgNPs for 24 h; LP: foliar spray of AgNPs at day 30; SLP: combined SP and LP. Data are means of 5 replicates. *p<0.05, **p<0.01, ***p<0.001.

Figures

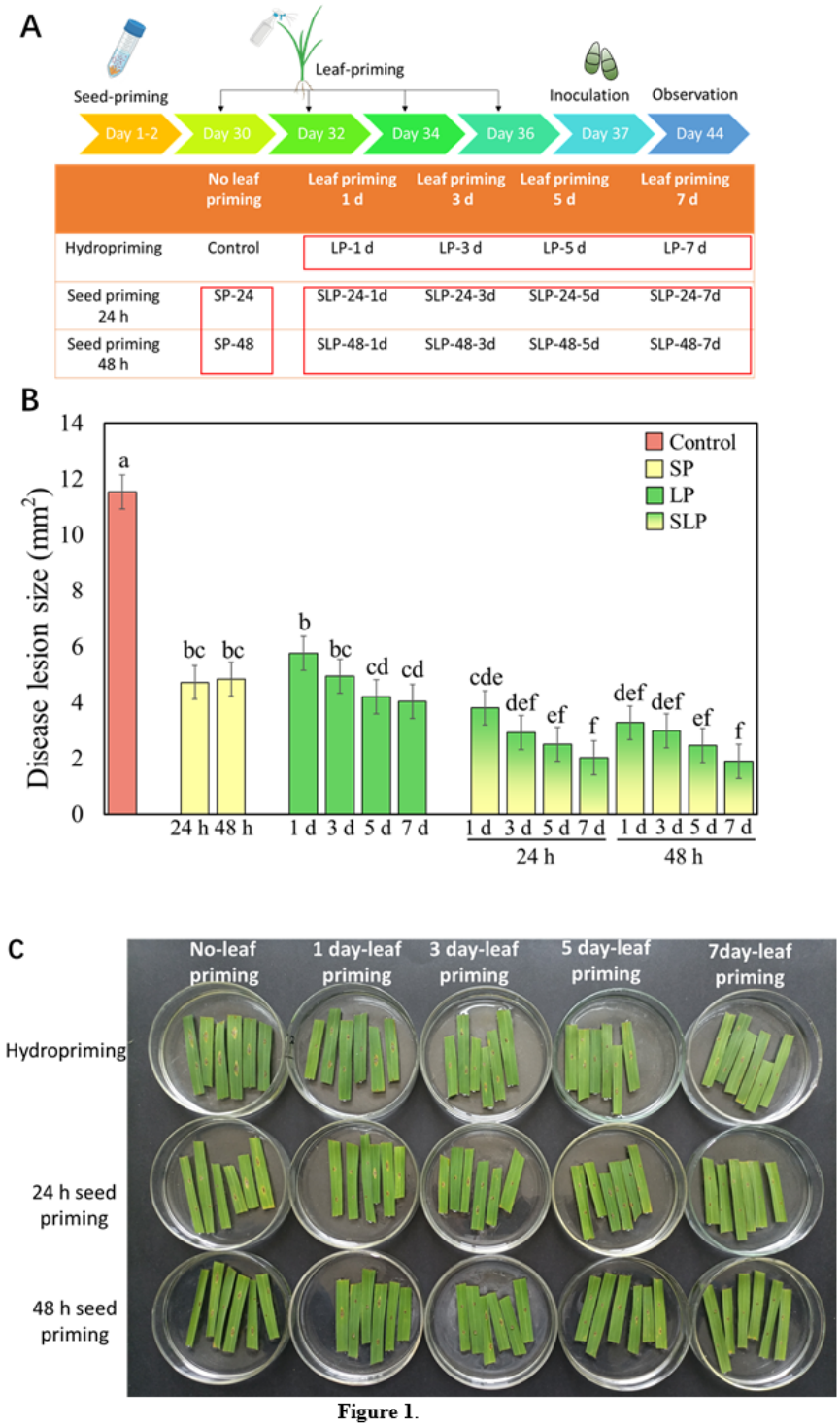

Figure 1.

Figure 1

(**A**) Schematic experimental design of AgNPs-based “stress training”. Three types of “stress training” were established, including seed-priming (SP) (seed treatment with 40 mg/L AgNPs for 24 or 48 h), leaf-priming (LP) (foliar spray of 40 mg/L AgNPs to rice seedlings at 1, 3, 5, and 7 d before stress exposure), and commbined seed- and leaf- priming (SLP), resulting in 15 treatments. The 37-day-old rice seedlings (training completed) were exposed to either rice blast or cold stress. (**B**) Disease lesions of detached rice

leaves (from 37-day-old trained rice seedlings) at 7 days post-inoculation with rice blast fungus *M. oryzae*. (**C**) Disease lesions sizes in rice leaves. Data are means ± s.d. (n=6). Different letters above bars represent differences ($P < 0.05$) determined by one-way analysis of variance (ANOVA) with Tukey's multiple compariso test.

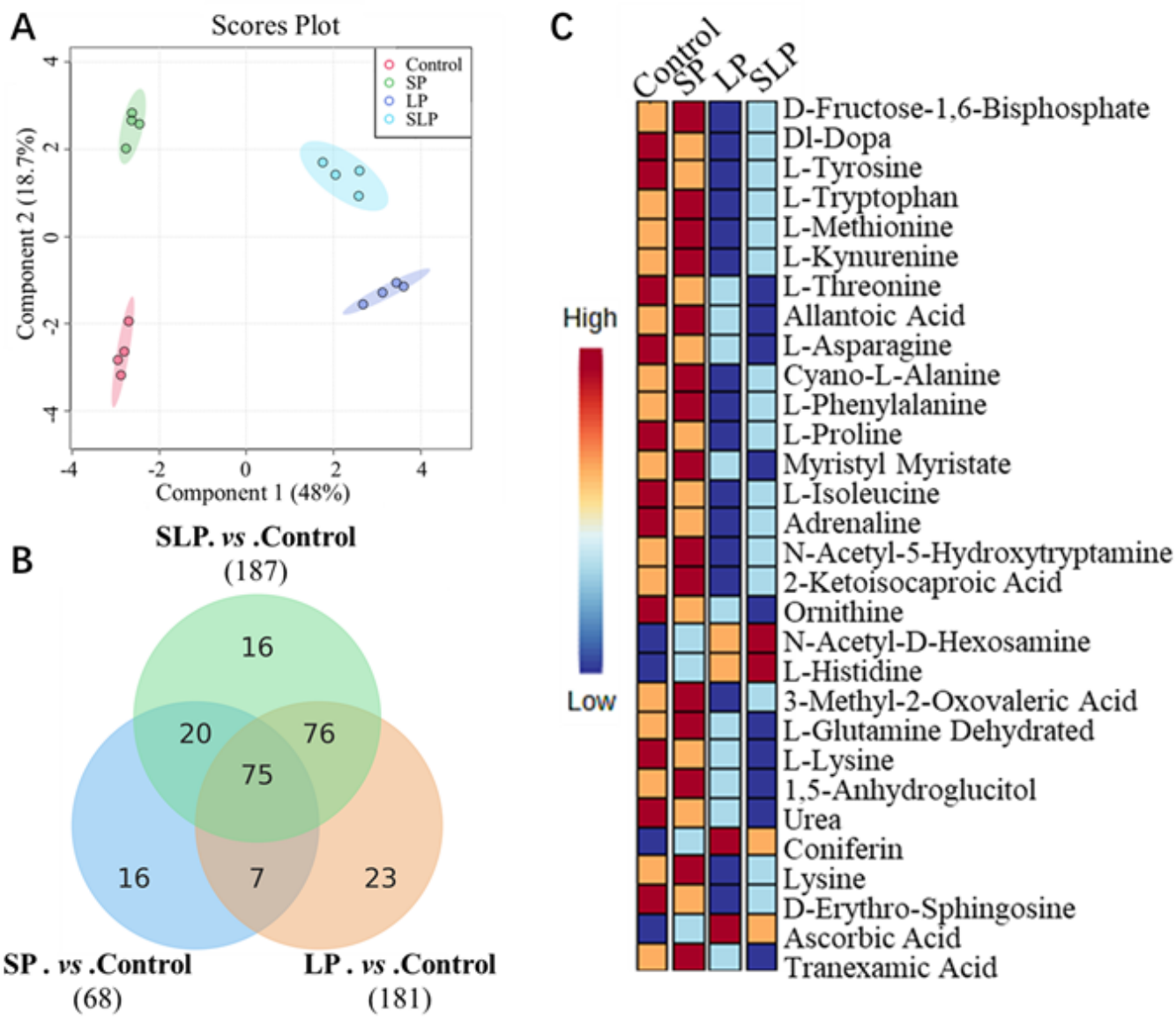

Figure 2.

Figure 2

Metabolite profile changes in trained rice leaves.(**A**) Sparse partial least-squares discriminate analysis (sPLS-DA) score plots of metabolic profiles in rice leaves without (control) and with "stress training" (SP, LP, SLP). (**B**) Venn diagram showing the number of changed metabolites in rice leaves with different

training (SP, LP, and SLP). (**C**) VIP score plot from sPLS-DA component 1, showing the metabolome pattern in four groups. Leaves subjected for metabolomics analysis are from 37-day-old trained rice seedlings. (**D**) KEGG pathway of rice leaves after SLP “stress training”.

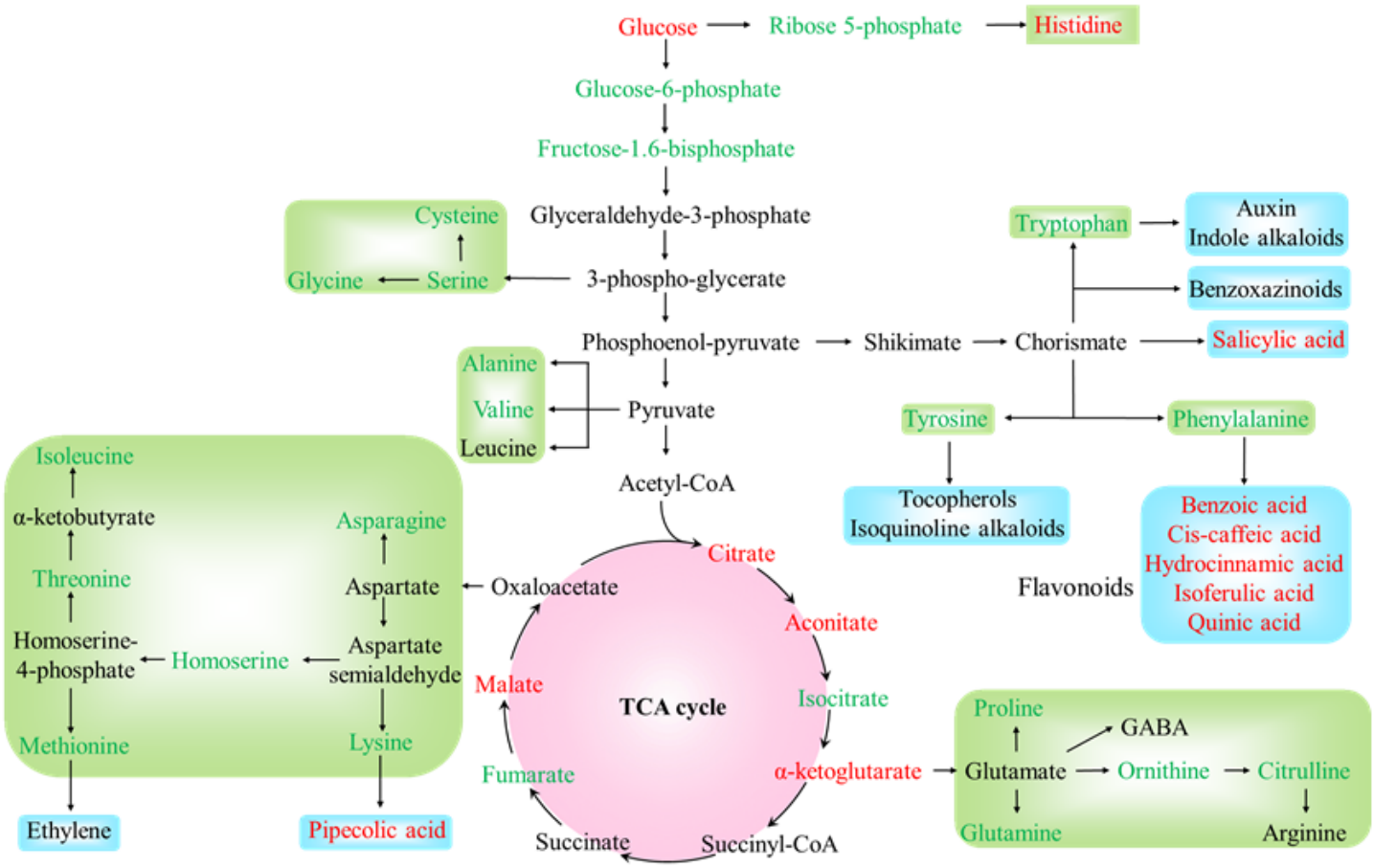

Figure 3.

Figure 3

Schematic of changed metabolic networks in rice leaves after SLP “stress training”. Pink represents TCA cycle, green represent amino acids metabolism pathways, blue represent specialized metabolites biosynthesis pathways. Red and green means the relative abundance of metabolites increased or decreased upon SLP training.

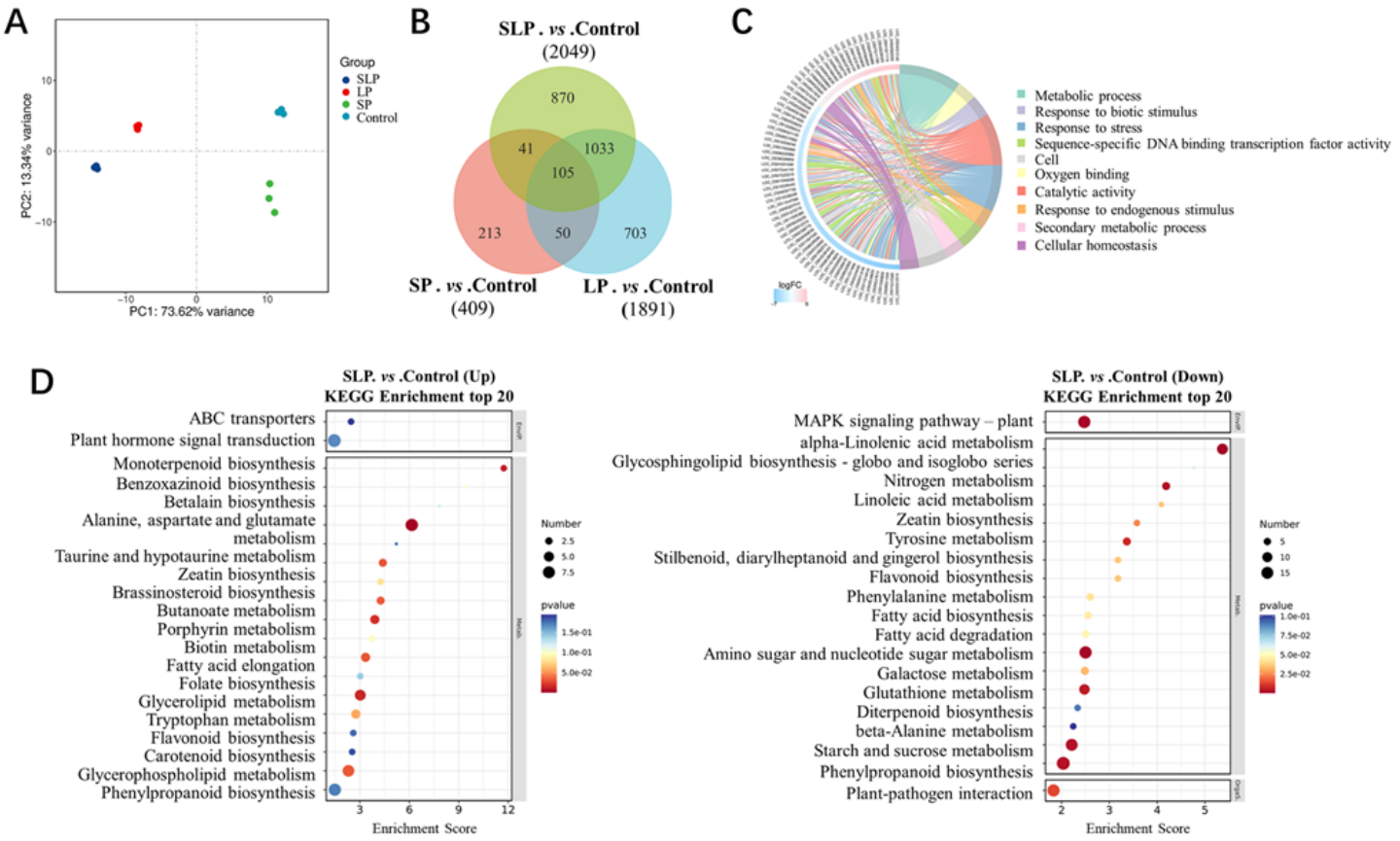

Figure 4

Figure 4

Transcriptome profile changes in rice leaves. (**A**) the principal component analysis (PCA) of transcriptome profiles of rice leaves. (**B**) Venn diagram showing the number of differentially expressed genes (DEGs), and the common and specific DEGs between different types of training (SP, LP, SLP). (**C**) Enriched Gene Ontology (GO) categories of 1000 genes by SLP stress training. (**D**) KEGG pathway enrichment analysis of DEGs induced by SLP training (left, up-regulated KEGGs; right, down-regulated KEGGs). Rice leaves subjected for RNA-seq analysis are from 37-day-old rice seedlings with SLP training or without training (control).

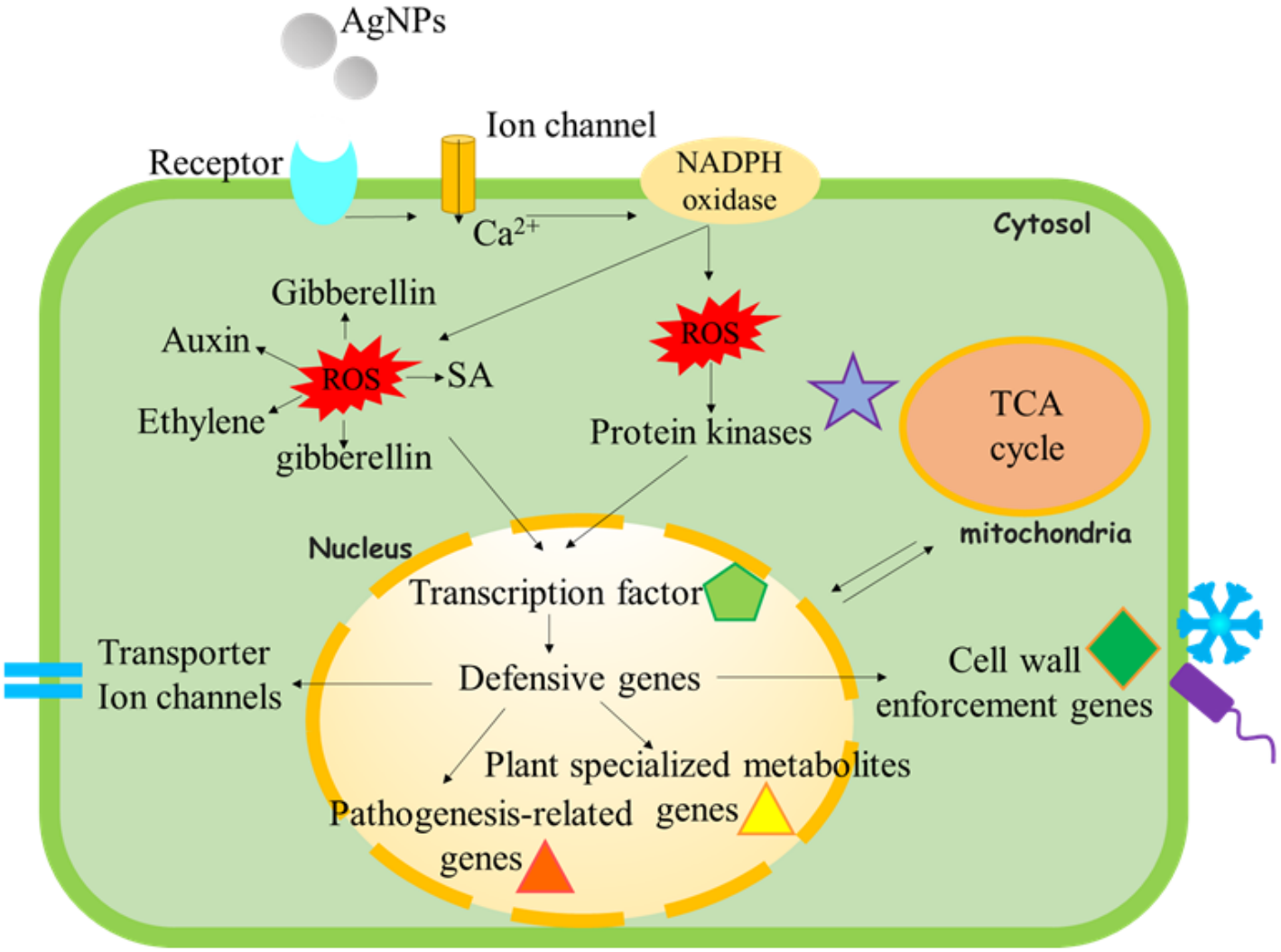

Figure 5.

Figure 5

Schematic of changed transcription networks in rice leaves with SLP “stress training”.

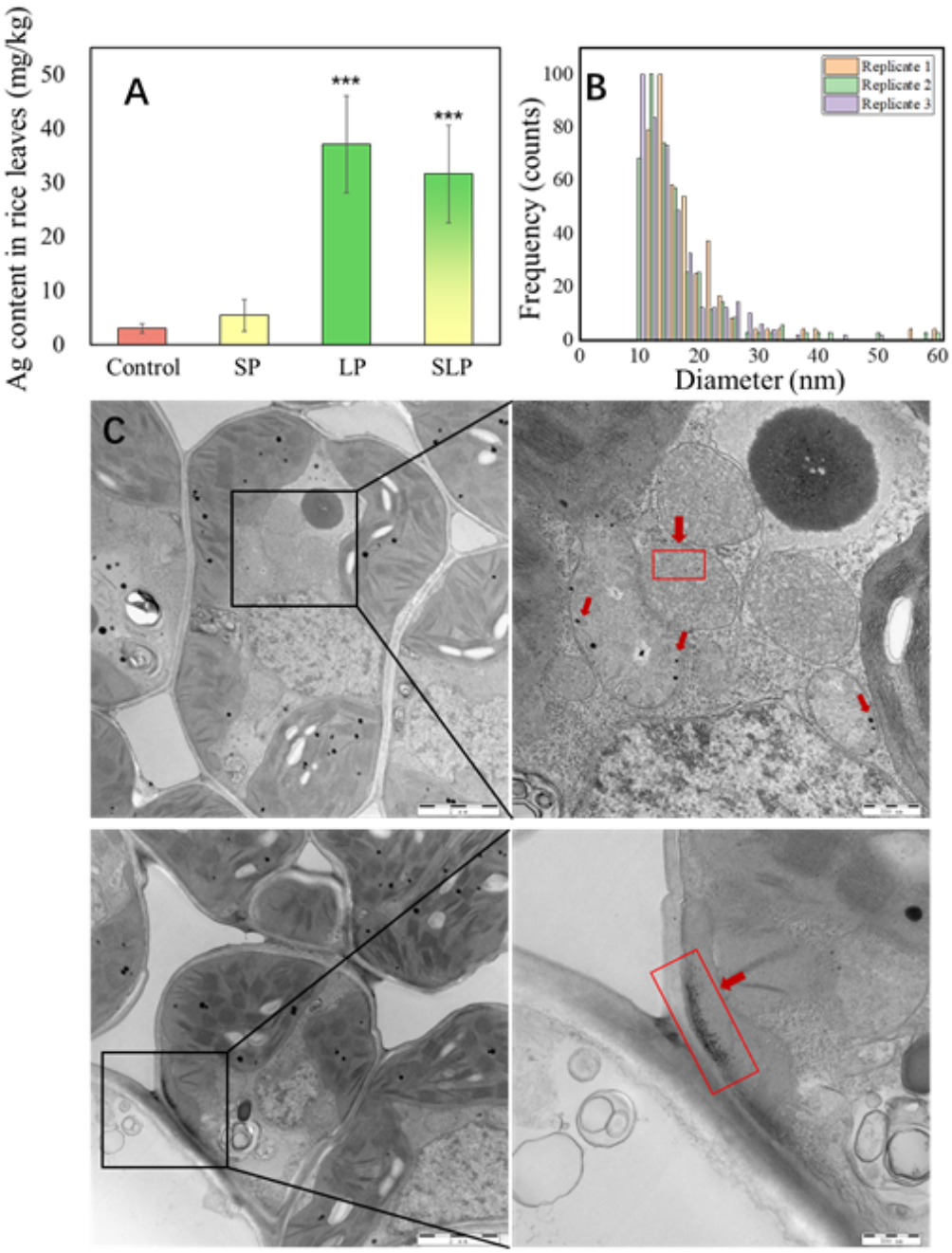

Figure 6.

Figure 6

AgNPs in rice leaves sprayed with 40 mg/L of AgNPs for 7 days. (**A**) ICP-MS results showing the Ag content in rice leaves. Data are means ± SD (n=6). (**B**) SP-ICP-MS data showing the amount of nanoparticulate Ag in rice leaves. (**C**) Representative TEM images of rice leaves cells 2 days post spray of 40 mg/L of AgNPs. The arrows indicate AgNPs are between cell walls and cytosol.

Supplementary Files

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