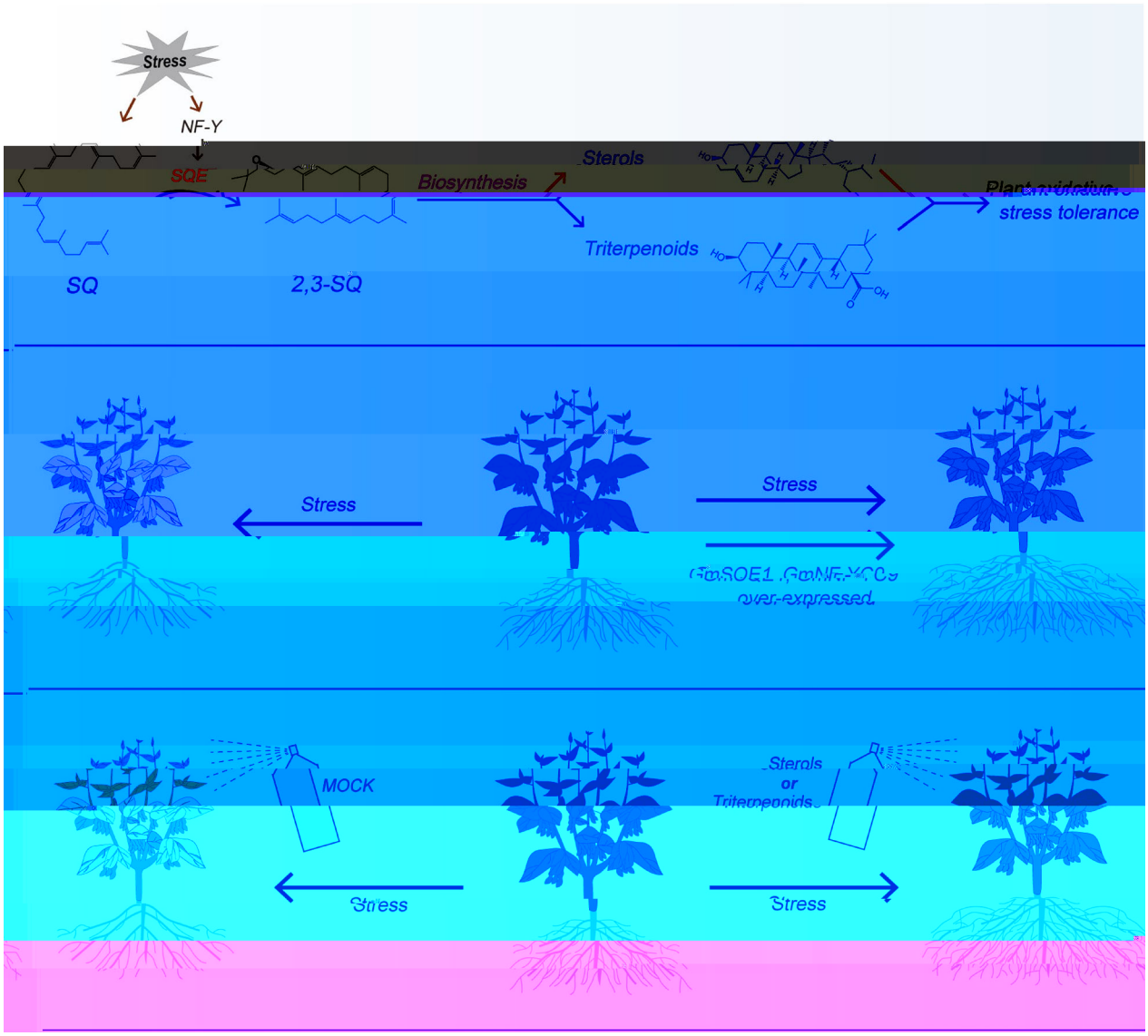


**Figure 7** The model of NF-Y-SQE module and soybean steroids improving plant stress tolerance. NF-Y-SQE module participates in regulating plant stress tolerance by enhancing SQE-mediated squalene metabolic pathways. Application of plant steroids or triterpenoids in crops enhanced their tolerance to drought stress.

### *In vitro* GST pull-down assay

The open reading frames (ORFs) of bait protein and candidate protein genes were cloned into the pCold™ TF vector (TaKaRa, Bio) and pGEX-4T-1 vector (GE Healthcare, Chicago, IL), respectively. Subsequently, the recombinant plasmids were introduced into the *E. coli* strain transetta (TransGen, Biotech). Expressions of the corresponding proteins were induced overnight using 0.5 mM isopropyl β-D-thiogalactoside (IPTG) (Inalco SPA, San Luis Obispo, CA) at 16 °C, and then a pull-down assay was performed. The experimental details were described by Yu *et al*. (2021).

### *In vitro* EMSA binding assay

The genes encoding three subunit proteins of the NF-Y transcription factor were cloned into expression vectors harbouring the tag proteins (His, GST, and MBP), respectively, and subsequently transferred into competent cells of the *E. coli* strain *Rosetta*. These recombinant proteins were employed for an EMSA using a biotin end-labelled duplex DNA probe. The method was previously described by Yu *et al*. (2021).

### Effect of fucosterol and soyasaponin II on crop growth under different treatments

Three-week-old of soybean, wheat, foxtail millet, and maize seedlings grown under normal conditions were subjected to controlled water treatment. During the treatment, the seedlings were sprayed with aqueous solutions of mock (40 mL distilled water containing 200 μL of 40 mM fatty alcohol polyoxyethylene ether (APE), which was a leaf surfactant), fucosterol (20 μL of fucosterol at a concentration of 600 mg/L was added into 40 mL distilled water containing 200 μL of 40 mM APE), and soyasaponin II (20 μL of soyasaponin II at a concentration of 600 mg/L was added into 40 mL distilled water containing 200 μL of 40 mM APE). The sprays were applied four times each week, with at least 1 day between each spraying. The changes in crop phenotypes

© 2024 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 1–15

14677652, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pbi.14349 by Institute of Crop Sciences, CA, Wiley Online Library on [26/04/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

were observed and recorded during controlled water treatment. The leaf samples of crops were collected at the onset of phenotypic variations in order to quantify physiological and biochemical alterations.

Five-week-old of soybean, wheat, foxtail millet, and maize seedlings cultivated under normal conditions were subjected to osmotic stress treatment by irrigating them with 200 mM mannitol aqueous solutions. During osmotic stress treatment, the seedlings were sprayed with mock (mock, 40 mL distilled water containing 200 μL of 40 mM APE), fucosterol (fucosterol aqueous solutions, 20 μL fucosterol (600 mg/L) into 40 mL distilled water containing 200 μL of 40 mM APE), and soyasaponin II aqueous solutions (soyasaponin II aqueous solutions, 20 μL soyasaponin II (600 mg/L) into 40 mL distilled water containing 200 μL of 40 mM APE). The sprays were applied four times each week, with at least 1 day between each spraying. The phenotype changes of the crops were observed and recorded during controlled water treatment. Leaf samples of the crops were taken when differences in the leave phenotype began to appear in order to measure physiological and biochemical changes.

### Metabolites extraction, UHPLC–MS–MS analysis, and data processing

The samples of stress induced WT and transgenic soybean seedlings (*GmSQE1*-OE6 and *GmNF-YC9*-OE8 plants) were freeze-dried, and then crushed with a mixer mill. A 50 mg aliquot of each individual samples were precisely weighed and transferred to Eppendorf tubes, which were used for metabolites extraction and UHPLC–MS analysis (Allwegene Technology Co., Ltd, Beijing, China). The methods of plant metabolites extraction were described in detail by Vos *et al*. (2007).

The UHPLC separation was performed using an EXIONLC System (Sciex). The mobile phase A was 0.1% formic acid in water, while mobile phase B was acetonitrile. The column temperature was set at 40 °C, and the auto-sampler temperature was set at 4 °C with an injection volume was 2 μL. A Sciex QTrap 6500+ (Sciex Technologies) was applied for assay development. Typical ion source parameters were as follows: IonSpray Voltage, +5500/−4500 V; Curtain Gas, 35 psi; Temperature, 400 °C; Ion Source Gas, 1:60 psi; Ion Source Gas 2, 60 psi; DP, ± 100 V (Allwegene Technology Co., Ltd, Beijing, China).

SCIEX Analyst Work Station Software (Version 1.6.3) was employed for MRM data acquisition and processing. MS raw data (.wiff) files were converted to the TXT format using MSconventer. An in-house R program and database were applied to peak detection and annotation (Allwegene Technology Co., Ltd, Beijing, China).

### Measurement of squalene, cycloartenol, and beta-amyrin by LC–MS

The samples of untreated, 25 mM terbinafine treated, and 100 mM mannitol induced soybean seedlings were freeze-dried, and then crushed with a mixer mill. A 50 mg aliquot of each sample was accurately weighed and transferred to Eppendorf tubes for metabolites extraction. The extracted metabolites were used for squalene, cycloartenol, and beta-amyrin content measurement by UHPLC–MS analysis. The method of extracting plant metabolites was described in detail by Vos *et al*. (2007).

Squalene (5 mg/mL) (Sigma, USA), cycloartenol (5 mg/mL) (Sigma, USA), and beta-amyrin (5 mg/mL) (Sigma, USA) were used in appropriate amounts to form standard solutions. These solutions were then diluted with methyl alcohol to create a suitable standard series of standards at concentrations of 5, 10, 20, 50, 100, 200, and 500 ng/mL. The peak areas of the standard substances at different concentrations were used to draw the standard curves that exhibited a good linear relationship with an $R^2$ value >0.99. The standard curves were drawn based on the peak areas of the standard substance at different concentrations and their corresponding concentrations. With an $R^2$ value >0.99, it indicated that the standard curves exhibit a strong linear relationship and could be utilised for calculating the concentrations of squalene, cycloartenol, and beta-amyrin in the samples.

## Acknowledgements

This research was financially supported by the National Key R & D Program of China (2022YFF1001600), China Postdoctoral Sci-

Cheng, Y., Wang, X., Cao, L., Ji, J., Liu, T. and Duan, K. (2021) Highly efficient *Agrobacterium rhizogenes*-mediated hairy root transformation for gene functional and gene editing analysis in soybean. *Plant Methods* **17**, 73.

Devireddy, A.R., Zandalinas, S.I., Fichman, Y. and Mittler, R. (2021) Integration of reactive oxygen species and hormone signaling during abiotic stress. *Plant J.* **105**, 459–476.

Dixon, S.J., Lemberg, K.M., Lamprecht, M.R., Skouta, R., Zaitsev, E.M., Gleason, C.E., Patel, D.N. *et al.* (2012) Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell* **149**, 1060–1072.

Forman, H.J. and Zhang, H. (2021) Targeting oxidative stress in disease: promise and limitations of antioxidant therapy. *Nat. Rev. Drug Discov.* **20**, 689–709.

Fujikawa, Y. and Kato, N. (2007) Split luciferase complementation assay to study protein-protein interactions in Arabidopsis protoplasts. *Plant J.* **52**, 185–195.

Garcia-Bermudez, J., Baudrier, L., Bayraktar, C., Shen, Y.S., La, K., Guarecuco, R., Yucel, B. *et al.* (2019) Squalene accumulation in cholesterol auxotrophic lymphomas prevents oxidative cell death. *Nature* **567**, 118–122.

He, J.X., Shozo, F., Li, T.C., Kang, S.G., Seto, H., Suguru, T., Shigeo, Y. *et al.* (2018) Sterols regulate development and gene expression in *Arabidopsis*. *Plant Physiol.* **131**, 2003.

Hou, X., Zhou, J., Liu, C., Liu, L., Shen, L. and Yu, H. (2014) Nuclear factor Y-mediated H3K27me3 demethylation of the SOC1 locus orchestrates flowering responses of Arabidopsis. *Nat. Commun.* **5**, 4601.

Jin, X., Li, X., Xie, Z., Sun, Y., Jin, L., Hu, T. and Huang, J. (2023) Nuclear factor OsNF-YC5 modulates rice seed germination by regulating synergistic hormone signaling. *Plant Physiol.* **193**, 2825–2847.

Kerchev, P.I. and Van Breusegem, F. (2022) Improving oxidative stress resilience in plants. *Plant J.* **109**

14677652, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pbi.14349 by Institute of Crop Sciences, CA, Wiley Online Library on [26/04/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Yoo, S.D., Cho, Y.H. and Sheen, J. (2007) *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat. Protoc.* **2**, 1565–1572.

Yoshioka, H., Coates, H.W., Chua, N.K., Hashimoto, Y. and Ohgane, K. (2020) A key mammalian cholesterol synthesis enzyme, squalene monooxygenase, is allosterically stabilized by its substrate. *Proc. Natl. Acad. Sci. USA* **117**, 15923–17158.

Yu, T.F., Ying Liu, Y., Fu, J.D., Ma, J., Fang, A.W., Chen, J., Zheng, L. *et al.* (2021) The NF-Y-PYR module integrates the abscisic acid signal pathway to regulate plant stress tolerance. *Plant Biotechnol. J.* **19**, 1–17.

Zandalinas, S.I., Fritschi, F.B. and Mittler, R. (2021) Global warming, climate change, and environmental pollution: recipe for a multifactorial stress combination disaster. *Trends Plant Sci.* **26**, 588–599.

Zanetti, M.E., Blanco, F.A., Beker, M.P., Battaglia, M. and Aguilar, O.M. (2010) A C subunit of the plant nuclear factor NF-Y required for rhizobial infection and nodule development affects partner selection in the common bean-Rhizobium etli symbiosis. *Plant Cell* **22**, 4142–4157.

Zhang, C., Zhang, H., Zhang, M., Lin, C., Wang, H., Yao, J., Wei, Q. *et al.* (2019) OSBPL2 deficiency upregulate SQLE expression increasing intracellular cholesterol and cholesteryl ester by AMPK/SP1 and SREBF2 signaling pathway. *Exp. Cell Res.* **383**, 111512.

Zhang, H., Zhu, J., Gong, Z. and Zhu, J.K. (2022) Abiotic stress responses in plants. *Nat. Rev. Genet.* **23**, 104–119.

Zhang, W.B., Tang, Y., Hu, Y.L., Yang, Y.H., Cai, J.J., Liu, H.L., Zhang, C.Y. *et al.* (2021) *Arabidopsis* NF-YCs play dual roles in repressing brassinosteroid biosynthesis and signaling during light-regulated hypocotyl elongation. *Plant Cell* **33**, 2360–2374.

Zhou, Y.Y., Zhang, Y., Wang, X.W., Han, X., An, Y., Lin, S.W., Shen, C. *et al.* (2020) Root-specific NF-Y family transcription factor, *PdNF-YB21*, positively regulates root growth and drought resistance by abscisic acid-mediated indoylacetic acid transport in Populus. *New Phytol.* **227**, 407–426.

Zhu, J.K. (2016) abiotic stress signaling and responses in plants. *Cell* **167**, 313–324.

## Supporting information

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

**Figure S1** Generation and stress tolerance identification of *GmNF-YC9*-OE soybean plants.

**Figure S2** Functional analysis of GmNF-YB24 and GmNF-YA2 candidate proteins.

**Figure S3** Differentially expressed genes of *GmNF-YC9*-OE soybean plants by transcriptome sequencing.

**Figure S4** Interaction analysis between the NF-Y transcription factor and the potential target gene *GmSQE1*.

**Figure S5** Effect of terbinafine on soybean plant growth and content analysis of squalene, cycloartenol, and β-amyrin under different treatments by LS-MS.

**Figure S6** Identification and stress tolerance identification of *GmSQE1* overexpressing soybean plants.

**Figure S7** Agronomic trait analysis of *GmNF-YC9*- and *GmSQE1*-OE soybean plants under normal conditions.

**Figure S8** Agronomic trait analysis of *GmSQE1*- and *GmNF-YC9*-OE soybean plants under drought conditions.

**Figure S9** Oxidative stress tolerance analysis of *GmNF-YC9*- and *GmSQE1*-OE soybean plants.

**Figure S10** Effect of terbinafine on soybean oxidative stress tolerance.

**Figure S11** Effect of fucosterol and soyasaponin II on crop stress tolerance.

**Figure S12** Effect of cycloartenol and beta-amyrin on crop stress tolerance.

**Figure S13** Expression level analysis of the genes related with response to ROS in *GmNF-YC9*-OE and WT soybean plants.

**Figure S14** Difference analysis of SQE-mediated catalytic pathway in humans and higher plants.

**Figure S15** Stress tolerance of soybean materials by application of rain-proof installations in the experimental field.

**Table S1**

14677652, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pbi.14349 by Institute of Crop Sciences, CA, Wiley Online Library on [26/04/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License