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VAMP726 from maize and *Arabidopsis* confers pollen resistance to heat and UV radiation by influencing lignin content of sporopollenin

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ABSTRACT

Sporopollenin in the pollen cell wall protects male gametophytes from stresses. Phenylpropanoid derivatives, including guaiacyl (G) lignin units, are known to be structural components of sporopollenin, but the exact composition of sporopollenin remains to be fully resolved. We analyzed the phenylpropanoid derivatives in sporopollenin from maize and *Arabidopsis* by thioacidolysis coupled with nuclear magnetic resonance (NMR) and gas chromatography–mass spectrometry (GC–MS). The NMR and GC–MS results confirmed the presence of *p*-hydroxyphenyl (H), G, and syringyl (S) lignin units in sporopollenin from maize and *Arabidopsis*. Strikingly, H units account for the majority of lignin monomers in sporopollenin from these species. We next performed a genome-wide association study to explore the genetic basis of maize sporopollenin composition and identified a vesicle-associated membrane protein (ZmVAMP726) that is strongly associated with lignin monomer composition of maize sporopollenin. Genetic manipulation of *VAMP726* affected not only lignin monomer composition in sporopollenin but also pollen resistance to heat and UV radiation in maize and *Arabidopsis*, indicating that VAMP726 is functionally conserved in monocot and dicot plants. Our work provides new insight into the lignin monomers that serve as structural components of sporopollenin and characterizes VAMP726, which affects sporopollenin composition and stress resistance in pollen.

Key words: pollen cell wall, sporopollenin, lignin monomers, heat stress, UV radiation

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INTRODUCTION

Pollen viability is vital to the yield of many cereal crops. The increasing frequency of extreme climate events severely affects pollen viability and leads to significant yield losses, posing great challenges to food security (Stapleton, 1992; Dolferus et al., 2011; Wheeler and von Braun, 2013; Chaturvedi et al., 2021). Plants have evolved the pollen cell wall to protect male gametophytes from unfavorable environmental conditions (Edlund et al., 2004). The exine is the outermost layer of the pollen cell wall; it consists mainly of sporopollenin, a biopolymer that is considered to be the toughest biomaterial in nature (Ariizumi and Toriyama, 2011). The intine is the inner cell wall

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layer deposited between the plasma membrane and the exine, and it has the typical composition of a primary cell wall (Jiang et al., 2013). For decades, the exact chemical composition of sporopollenin has remained elusive. Use of thioacidolysis coupled with solid-state nuclear magnetic resonance (NMR) analysis recently revealed that sporopollenin consists mainly of crosslinked long-chain polyvinyl alcohol units and coumaroylated C16 aliphatic units (Li et al., 2019). Phenylpropanoid derivatives, including naringenin, *p*-hydroxybenzoate (*p*-BA), *p*-coumarate (*p*-CA), ferulate (FA), and guaiacyl (G) lignin units, are also present in sporopollenin (Li et al., 2019; Xue et al., 2020).

Phenylpropanoids comprise a wide variety of secondary metabolites that originate from phenylalanine (Fraser and Chapple, 2011). These aromatic compounds are essential for plant growth, development, and adaptive responses to environmental changes (Dong and Lin, 2021). For example, phenylpropanoid derivatives in sporopollenin can confer pollen resistance to ultraviolet (UV) radiation (Xue et al., 2020). Lignin is an important polymeric phenylpropanoid derivative that is mainly deposited in the secondary cell wall to provide mechanical strength and hydrophobicity (Bonawitz and Chapple, 2010); it also provides other benefits, such as resistance to biotic and abiotic stresses (Cesarino, 2019). Formation of the lignin polymer requires three major monolignols, *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. Once these monolignols enter the apoplastic space, they are activated by cell-wall-bound oxidation systems, initiating radical coupling to form lignin polymers (Bonawitz and Chapple, 2010). Accordingly, lignin polymers typically consist of the three most abundant monomers, *p*-hydroxyphenyl (H), G, and syringyl (S) lignin units, which are derived from *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, respectively (Bonawitz and Chapple, 2010). Different plant species have distinct lignin monomer compositions, and lignin also exhibits tissue-specific differences in composition within the same plant (Campbell and Sederoff, 1996). Interestingly, only G units were detected in sporopollenin of *Cryptomeria* (gymnosperm) and *Lilium* (angiosperm), and no lignin units were detected in sporopollenin of two seedless plants, *Ophioglossum* (fern) and *Lycopodium* (moss) (Xue et al., 2020), indicating that monolignol biosynthesis may have been involved in the environmental adaptation of early vascular plants.

Sporopollenin precursors are synthesized in the tapetum, a specific cell layer that surrounds the developing pollen grains (Wang et al., 2018). The absence of S units in sporopollenin of tested plant species is likely due to the low expression of genes encoding enzymes required for sinapyl alcohol biosynthesis in the tapetum, such as caffeic acid *O*-methyltransferase (COMT) and ferulic acid 5-hydroxylase (F5H) (Bonawitz and Chapple, 2010; Xue et al., 2020). Once synthesized, these precursors are translocated from the tapetal cells to the locules between the tapetum and immature pollen grains, then incorporated into primexine (the surface of the developing pollen grains) to form the exine (Shi et al., 2015). Transport proteins, such as ATP-binding-cassette transporters and lipid-transfer proteins, have been proposed to mediate delivery of sporopollenin precursors across the plasma membrane (Zhang et al., 2010; Choi et al., 2011, 2014; Huang et al., 2013; Quilichini et al., 2014). Secretory vesicles derived from the endoplasmic reticulum-*trans*-Golgi network might also be involved in the secretion of sporopollenin precursors (Ichino and Yazaki, 2022).

In this study, we analyzed the phenylpropanoid derivatives in sporopollenin from *Zea mays* (hereafter maize) and *Arabidopsis thaliana* (hereafter *Arabidopsis*). We found that H, G, and S lignin units are structural components of maize and *Arabidopsis* sporopollenin, and H units is much more abundant than the other two monomers. We confirmed that the monolignol biosynthetic pathway is required for pollen resistance to heat and UV stresses and identified vesicle-associated membrane protein 726 (VAMP726), which positively influences lignin monomer composition in sporopollenin, through a genome-wide association study (GWAS). Genetic manipulation of *VAMP726* expression strongly affected pollen resistance to heat and UV radiation in both maize and *Arabidopsis*. Finally, we showed that overexpression of *VAMP726* resulted in enrichment of proteins related to lignification in the apoplastic space. This work reveals a metabolic strategy deployed by plants to enhance pollen stress resistance.

RESULTS

H units comprise the majority of lignin monomers in sporopollenin from maize and *Arabidopsis*

Several phenylpropanoid derivatives, such as *p*-BA, *p*-CA, FA, naringenin, and G lignin units, have been detected in thioacidolysis lysates of sporopollenin from vascular plants (Li et al., 2019; Xue et al., 2020). The exclusive presence of G lignin units, but not other major lignin monomers, in sporopollenin attracted our attention. Gymnosperm lignin is composed mainly of G units, whereas angiosperms mostly produce G- and S-type lignin (Ralph et al., 2019). The absence of S and H lignin units in sporopollenin of *Cryptomeria* (gymnosperm) and *Lilium* (dicot) (Xue et al., 2020) caused us to wonder whether "G-lignin-only" sporopollenin is a universal phenomenon across the plant kingdom. To test this possibility, we collected pollen grains from field-grown maize, one of the most important monocot cereal crops, and prepared sporopollenin (Figure 1A). We then analyzed the presence of lignin monomers and other phenylpropanoid derivatives in the thioacidolysis lysate of sporopollenin by solution-state two-dimensional (2D)-NMR spectroscopy. The thioacidolysis products of maize sporopollenin yielded typical NMR signal patterns of H and G lignin units, suggesting that, in addition to G units, H units are also present in maize sporopollenin (Figure 1B). G and S lignin units comprise the vast majority of lignin monomers in most lignified tissues of maize (Kim et al., 2017; Del Rio et al., 2018; Sun et al., 2018). To compare lignin monomer composition between maize sporopollenin and other maize tissues, we analyzed the lignin monomer composition of a mixture of tissues (stem, leaf, and leaf sheath) by NMR. The thioacidolysis products of these maize tissues exhibited a typical lignin monomer composition consisting of H, G, and S units (Figure 1B). Because *p*-BA and *p*-CA can yield signature features of H units, they might interfere with the detection of H lignin units derived from *p*-coumaryl alcohol, resulting in an exaggerated level of this specific monomer (Ralph et al., 2019). To rule out the possibility that thioacidolysis breaks down *p*-BA or *p*-CA, we looked for the NMR signal patterns of these

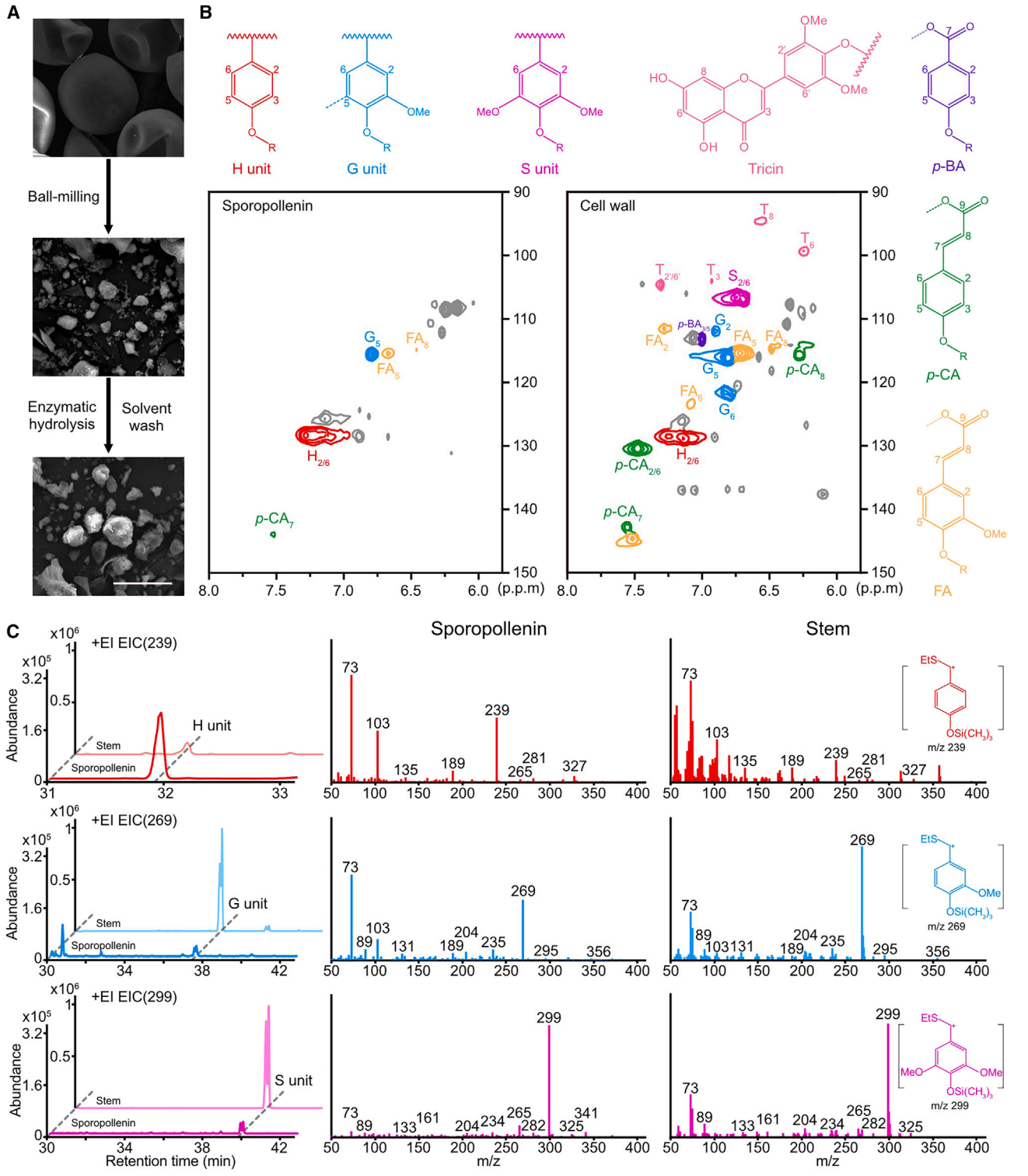

Figure 1. H, G, and S lignin units are structural components of maize sporopollenin.

(A) Electron micrographs of maize pollen grains (top), the fine powder after ball milling (middle), and the destarched extractive-free cell-wall residues of maize pollen (bottom). Scale bar, 500 μm.

(B) 2D-NMR spectra of thioacidolysis-released products of maize sporopollenin and cell walls extracted from a mixture of lignified maize tissues (stem, leaf, and leaf sheath), showing the aromatic signals of H, G, and S lignin units, as well as tricin, *p*-BA, *p*-CA, and FA.

(C) Selected ion chromatograms and MS^2 spectra of the indicated lignin monomers in thioacidolysis-released products of maize sporopollenin or maize stems from GC–MS. The structures of characteristic base peak fragment ions are shown. Results obtained from the maize inbred line MN are used here for representation.

phenolics in the thioacidolysis-released fractions of maize sporopollenin and cell-wall residues from other tissues. The results confirmed the presence of *p*-CA and FA in maize sporopollenin, and all three phenolics were present in other maize tissues (Figure 1B), suggesting that H units detected in sporopollenin by NMR are not derived from *p*-BA or *p*-CA. Tricin is a flavonoid that is incorporated as a lignin monomer into the lignin polymers of monocot plants (Lan et al., 2015). We also identified the presence of tricin in the NMR spectra of mixed maize tissues but not of sporopollenin (Figure 1B).

To validate the NMR results, we used gas chromatography–mass spectrometry (GC–MS) to determine the presence of each lignin monomer in maize sporopollenin. In this case, we performed a population-wide investigation of lignin monomer composition in sporopollenin from 117 field-grown maize inbred lines (Supplemental Dataset 1). In all of these lines, we detected the characteristic chromatograms and mass fragment ions of H, G, and S units in sporopollenin (Figure 1C), which were mostly identical to those of synthesized standards (Yue et al., 2012) and to lignin monomers detected in the xylem (maize stem, Figure 1C). We wondered whether pollen grains of a dicotyledonous plant, e.g., *Arabidopsis*, would be encased by sporopollenin with a similar lignin monomer composition, as dicots typically contain few H lignin units (Ralph et al., 2019). We therefore analyzed the lignin monomer composition of *Arabidopsis* sporopollenin by thioacidolysis coupled with GC–MS. The results showed that *Arabidopsis* sporopollenin also contained H, G, and S lignin units (Supplemental Figure 1). Moreover, we were able to detect the presence of *p*-BA, *p*-CA, and FA in maize and *Arabidopsis* sporopollenin through GC–MS (Supplemental Figure 2). Although we did not detect the NMR signals of S units in sporopollenin, this may be because the abundance of S units in the sample prepared for NMR analysis was too low to be captured. Nonetheless, combining the results from NMR and GC–MS, we conclude that H, G, and S lignin units are structural components of maize and *Arabidopsis* sporopollenin.

The presence of *p*-BA, *p*-CA, and FA, as well as three major lignin monomers, in sporopollenin prompted us to investigate how they integrate into sporopollenin. Thioacidolysis is routinely used in the analysis of lignin monomer composition of the plant cell wall; it mainly breaks ether bonds and ester bonds between different lignin monomers (Eudes et al., 2012; Ralph et al., 2019). We wondered whether these phenylpropanoid derivatives could be attached to the aliphatic units of sporopollenin through ester bonds. Hence, we used methanol or NaOH treatment during preparation of maize sporopollenin to release soluble phenolics and wall-bound phenolics (through ester bonds) from the pollen cell wall, then subjected the remaining residues to thioacidolysis (Supplemental Figure 3A). We found that *p*-CA was present in the wall-bound phenolics but not in the soluble fractions (Supplemental Figure 3B). FA and *p*-BA were detected in both wall-bound and soluble phenolics (Supplemental Figure 3B). These results confirmed that these phenolics could be attached to metabolite units (lignin monomers or aliphatic units) in maize sporopollenin through ester bonds. In addition, we did not detect any signal of lignin monomers in these washing fractions (Supplemental Figure 3B), and we could still detect all three major lignin monomers in the thioacidolysis-released products (Supplemental Figure 3C), suggesting that these lignin monomers were not attached to metabolite units of maize sporopollenin through ester bonds. These data indicate that the lignin monomers detected in the thioacidolysis-released fractions of sporopollenin likely form a lignin polymer or crosslink with some other metabolite units of sporopollenin through ether bonds. More sophisticated analytical methods will be required to dissect the exact linkage types.

We quantified the absolute abundance of each lignin unit in sporopollenin from each maize inbred line on the basis of peak areas in the GC–MS chromatograms. For simplicity, we defined the total content of lignin monomers as the sum of the abundance of H, G, and S units. We found that, at a population scale, the overall H units accounted for more than 70% of lignin monomers in maize sporopollenin, and no difference was observed between the abundance of G and S units (Figure 2A and 2B). This is surprising, because H units usually represent a minor constituent (less than 30%) of total lignin monomers in most lignified plant tissues (Ralph et al., 2019). In *Arabidopsis* sporopollenin, the abundance of H units was highest (accounting for more than half of total lignin monomers) and that of S units was lowest (Supplemental Figure 4). To ensure the reliability of the analytical methods, we analyzed the lignin monomer composition of other tissues from maize cultivar B73, including the leaf, stem, tassel (male flowers were removed), glume, and leaf sheath. We detected an extremely high amount of H units in B73 sporopollenin, significantly higher than that of G and S units (Figure 2C). In other tissues of B73, lignin consisted mainly of G and S units, and H units accounted for less than 2% of total lignin monomers (Figure 2D), consistent with previous reports (Kim et al., 2017; Del Rio et al., 2018; Sun et al., 2018). We also noticed that the total yield of lignin monomers from other tissues was at least 20 times that from sporopollenin (Figure 2C and 2D). Collectively, these results confirmed that H, G, and S lignin units are present in sporopollenin of maize and *Arabidopsis* and that H units are the dominant lignin monomers in sporopollenin.

The monolignol biosynthetic pathway is required for lignin monomer accumulation in sporopollenin and pollen stress resistance in *Arabidopsis*

The maize population we used for analysis of lignin monomer composition could be divided into two groups on the basis of geographic origin (Supplemental Dataset 1). Interestingly, the total content of lignin monomers in sporopollenin was significantly higher in maize inbred lines from tropical/subtropical areas than in those from temperate areas (Figure 3A), suggesting that lignin monomers in sporopollenin may be important for maize adaptation to local environmental conditions. The UV-absorbing property of phenylpropanoid derivatives could protect plants from damage caused by excess exposure to solar radiation (Dixon and Paiva, 1995; Qian et al., 2015). In *Arabidopsis*, mutations in key enzymes of monolignol biosynthesis, such as cinnamate-4-hydroxylase (C4H) and cinnamoyl-coenzyme A (CoA) reductase (CCR), resulted in defective sporopollenin and hypersensitivity of pollen to UV radiation (Xue et al., 2020). In the field, maize pollen grains are often exposed to high temperatures and high doses of UV during summer periods, both of which could markedly affect pollen viability. We wondered whether lignin monomer composition of maize sporopollenin is associated with

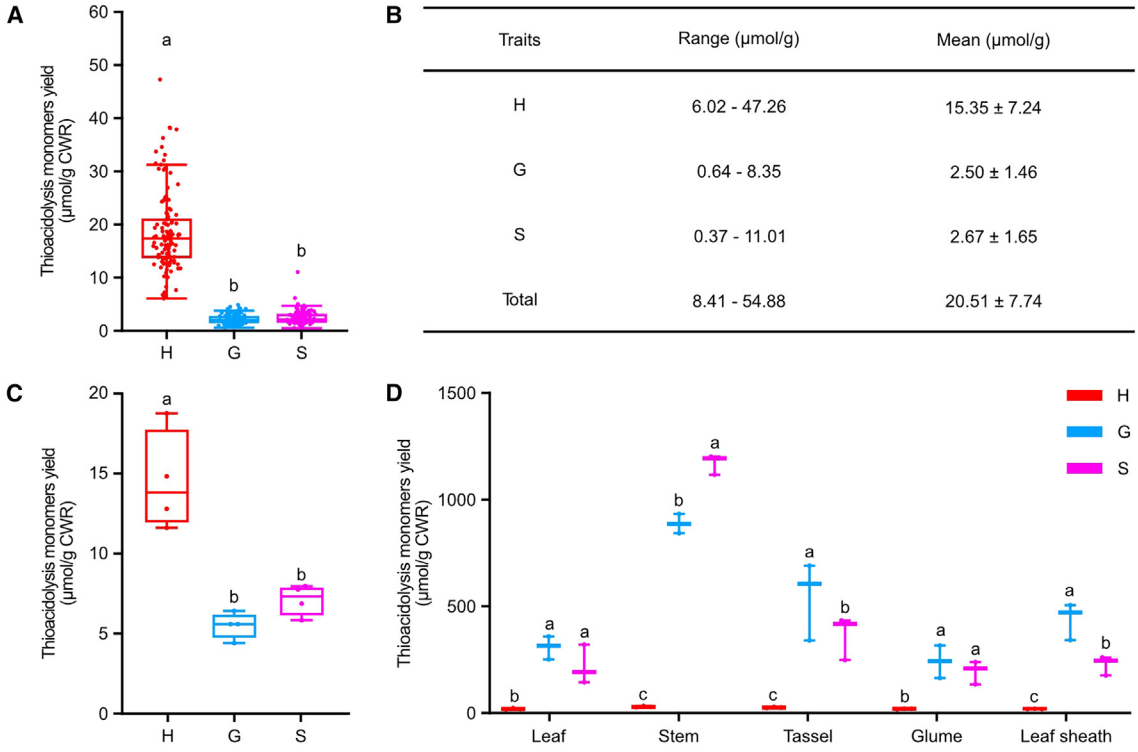

Figure 2. H units comprise the majority of lignin monomers in maize sporopollenin.

(A) Quantification of lignin monomers in thioacidolysis-released products of sporopollenin from 117 maize inbred lines. One-way ANOVA followed by Tukey's honestly significant difference test was used for statistical analysis (n = 117, P < 0.05). Error bars represent SD. Letters indicate significant differences.

(B) Descriptive statistics for lignin monomer composition in sporopollenin of 117 maize inbred lines.

(C) Quantification of lignin monomers in thioacidolysis-released products of sporopollenin from B73. One-way ANOVA followed by Tukey's honestly significant difference test was used for statistical analysis (n = 4, P < 0.05). Error bars represent SD. Letters indicate significant differences.

(D) Quantification of lignin monomers in thioacidolysis-released products of leaf, stem, tassel, glume, and leaf sheath tissues of B73. Two-way ANOVA followed by Tukey's honestly significant difference test was used for statistical analysis (n = 4, P < 0.05). Error bars represent SD. Letters indicate significant differences in the abundance of individual monomers within each tissue.

resistance of pollen to heat. Therefore, we harvested pollen grains of selected maize inbred lines that contained the highest or lowest levels of total lignin monomers in sporopollenin (Supplemental Dataset 2) and examined their pollen viability under heat treatment using triphenyl tetrazolium chloride (TTC) staining (Alexander, 1969). The results showed that maize inbred lines that contained more lignin monomers in sporopollenin overall produced pollen grains that were better able to cope with heat stress (Figure 3B and Supplemental Figure 5), suggesting that lignin monomers in sporopollenin may contribute to pollen heat resistance.

We speculated that deficiency in monolignol biosynthesis would affect lignin monomer content in sporopollenin and lead to impaired pollen resistance to stresses, including heat. To test this possibility, we took advantage of well-studied *Arabidopsis* mutants (*ccr1*, *comt*, and *ccomt*) that are defective in monolignol biosynthesis. CCR, COMT, and caffeoyl-CoA 3-*O*-methyltransferase (which was mutated in *ccomt*) all catalyze critical steps in monolignol biosynthesis (Bonawitz and Chapple, 2010), and mutations in these genes significantly alter lignin monomer composition in *Arabidopsis* (Do et al., 2007; Rinaldi et al., 2016). We used *tt4*, in which chalcone synthase (CHS) is mutated, as a control, because lack of CHS would block the biosynthesis of flavonoids but not of lignin (Li et al., 2010). We first examined whether pollen grains from these mutants were more prone to heat stress. We used germination rate as an alternative metric to assess pollen viability because the TTC staining protocol did not work well for *Arabidopsis* pollen. The results showed that *ccr1*, *comt*, and *ccomt* pollen had significantly lower resistance to heat (Figure 3C). The *tt4* mutant also exhibited moderately reduced heat resistance, but to a much lesser extent (Figure 3C). After exposure to high temperature for 30 min, pollen resistance of *tt4* did not differ significantly from that of Columbia-0 (Col-0) and was only moderately reduced when the treatment was extended to 60 min (Figure 3C).

We next investigated whether lignin monomer content in sporopollenin of these mutants was altered as predicted. The results showed that abundance of H units in sporopollenin was not significantly different in *ccr1*, but it was significantly lower in *ccomt* than in Col-0 (Supplemental Figure 6). The abundance of G and S units in sporopollenin of *ccr1* and *ccomt* did not change significantly (Supplemental Figure 6). However, the abundance of total lignin monomers in sporopollenin was significantly lower in *ccr1 and ccomt* than in Col-0 (Supplemental Figure 6). These results suggest that mutations in monolignol biosynthesis negatively affect the lignin monomer

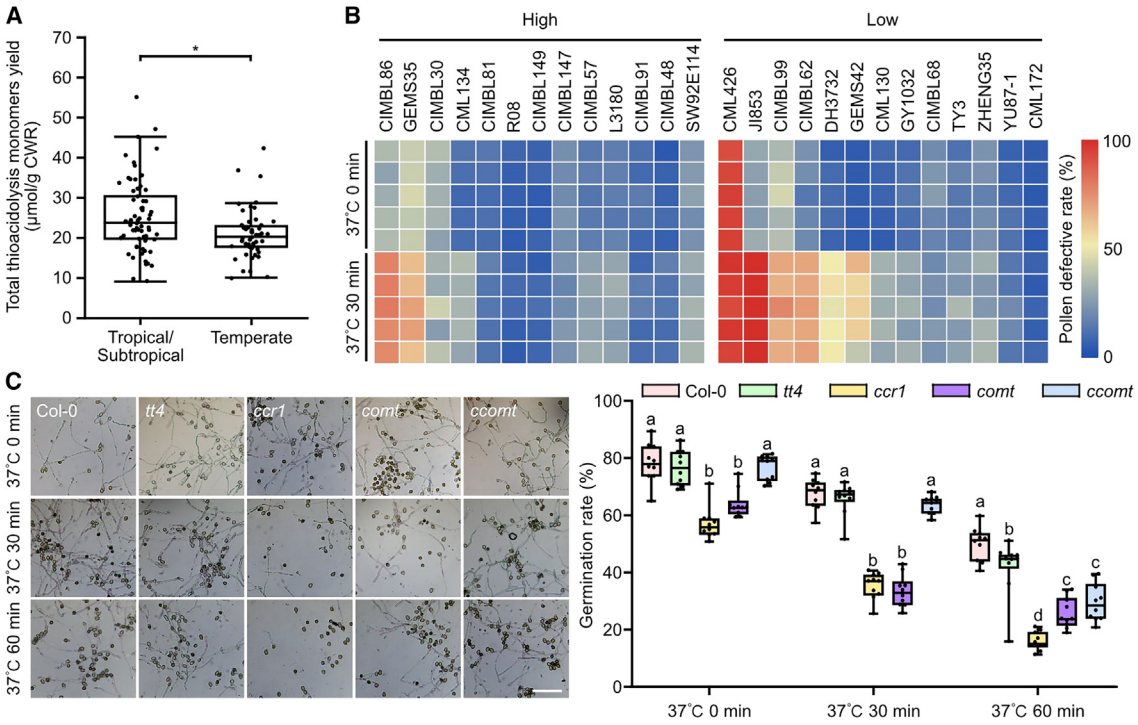

Figure 3. Monolignol biosynthesis is essential for pollen resistance to stresses in *Arabidopsis*.

(A) Comparison of total lignin monomers in thioacidolysis-released products of sporopollenin from maize inbred lines originating from tropical/subtropical and temperate areas. Student's *t*-test was used for statistical analysis (tropical/subtropical, $n = 65$; temperate, $n = 52$; $P < 0.05$). Error bars represent SD. Asterisk indicates a significant difference.

(B) Heatmap showing the percentage of defective pollen in inbred lines with the highest and lowest levels of total lignin monomers in sporopollenin before and after heat treatment. Five biological replicates (shown in five rows) were analyzed for each inbred line.

(C) Pollen viability of *Arabidopsis* before and after heat treatment. Fresh pollen grains were exposed to 37°C for the indicated times and then tested for *in vitro* germination. Scale bar represents 200 μm. Two-way ANOVA followed by Tukey's honestly significant difference test was used for statistical analysis ($n = 10$, $P < 0.05$). Error bars represent SD. Letters indicate significant differences in germination rate at each time point.

content of *Arabidopsis* sporopollenin. We also analyzed lignin monomer composition in sporopollenin of *tt4* and found no significant differences in the abundance of individual units or total lignin monomers compared with Col-0 (Supplemental Figure 6), consistent with its stronger pollen heat resistance compared with the other mutants (Figure 3C). On the basis of our data and previous observations of reduced pollen resistance to UV radiation in *c4h* and *ccr1* (Xue et al., 2020), we concluded that the monolignol biosynthetic pathway is required for lignin monomer accumulation in sporopollenin and pollen resistance to heat and UV radiation in *Arabidopsis*.

Natural variation in *ZmVAMP726* is associated with lignin monomer composition in maize sporopollenin

The essential role of monolignol biosynthesis in pollen stress resistance prompted us to investigate the genetic basis for lignin monomer composition in maize sporopollenin. The genetic diversity of 117 maize inbred lines enabled us to perform a GWAS using the total content of lignin monomers in sporopollenin as the target trait. Using a mixed linear model, we detected two adjacent SNPs, chr9.S_153426472 and chr9.S_153426479, that exceeded the significance threshold for association (Figures 4A and 7A; Supplemental Dataset 3). We performed an additional GWAS using the abundance of H units in sporopollenin as the target trait in order to explore the potential mechanism by which maize deposits this prominent lignin monomer in sporopollenin. Interestingly, the two GWASs revealed identical significant SNPs (Supplemental Figure 7B and Supplemental Dataset 3), suggesting that the variation in total content of lignin monomers in maize sporopollenin is likely driven by variation in the abundance of H units. We designated the 60-kb (±30-kb) genomic region surrounding each SNP as a quantitative trait locus (QTL). The two QTLs almost completely overlapped and explained over 30% of the phenotypic variation (Supplemental Dataset 3). Six candidate genes were present in this region (Supplemental Dataset 3), and we examined their developmental expression patterns using the Maize eFP Browser (Hoopes et al., 2019). Finally, *GRMZM2G075588* was selected for further analysis, as it was the only gene specifically expressed in anthers (Supplemental Figure 8).

GRMZM2G075588 is predicted to encode a VAMP. VAMPs belong to a subgroup of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), which mainly mediate vesicle trafficking of proteins or metabolites via membrane fusion (Sanderfoot et al., 2000). The protein sequence encoded by *GRMZM2G075588* shares 74.1% amino acid identity with VAMP726 in *Arabidopsis* (Figure 4B), and we therefore designated it *ZmVAMP726*. Expression analysis by quantitative real-time PCR confirmed that *GRMZM2G075588* was expressed mainly in tassels (Figure 4C), suggesting a

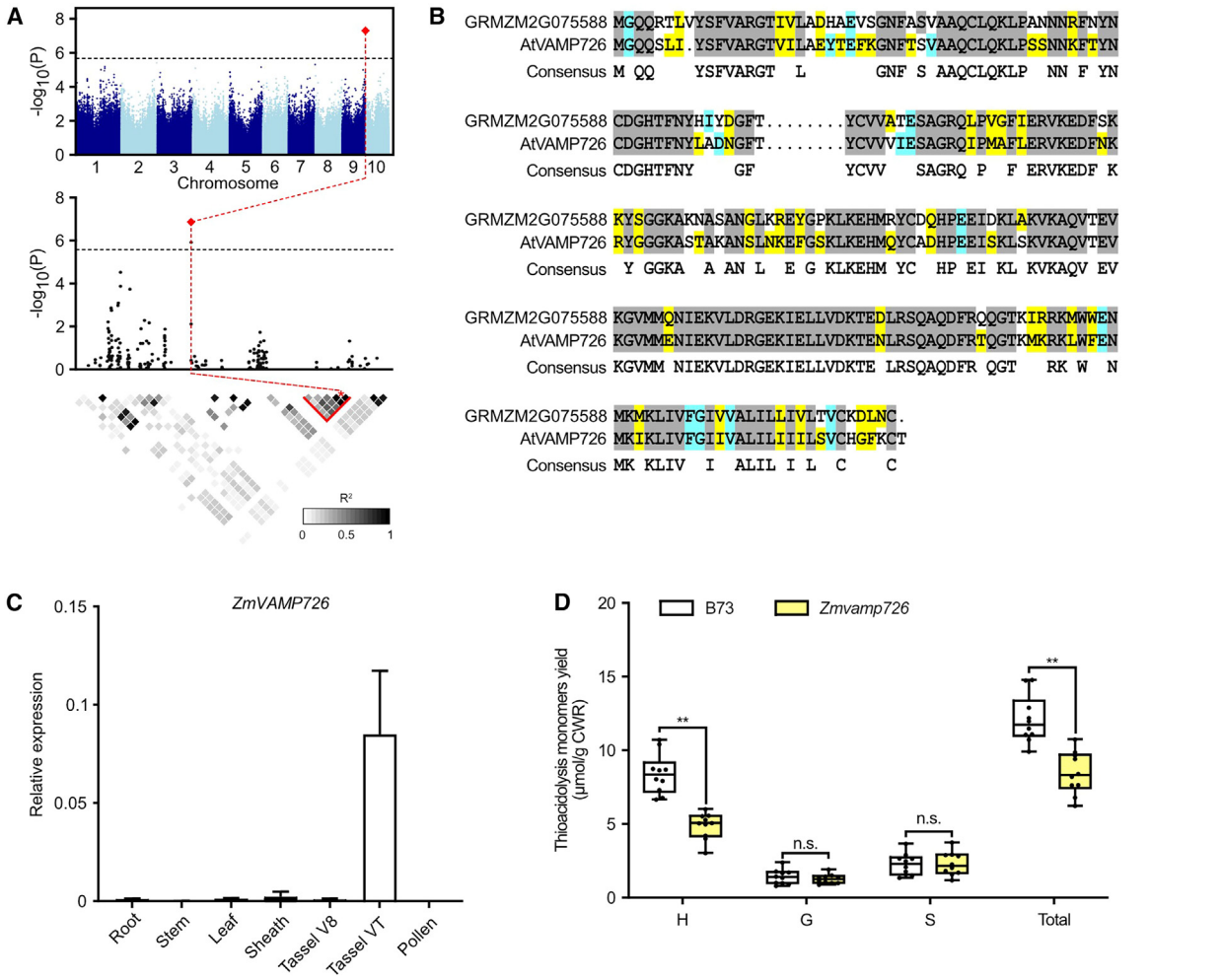

Figure 4. Natural variation in *ZmVAMP726* is associated with lignin monomer composition in maize sporopollenin.

(A) GWAS results for total lignin monomer content in thioacidolysis-released products of maize sporopollenin. The lead SNP is highlighted in red. The window (middle) represents the 60-kb genomic region surrounding the most significant SNP. The LD heatmap (bottom) shows pairwise R^2 values between polymorphisms in the 60-kb region centered on the most significant SNP.

(B) Protein sequence alignment of AtVAMP726 and GRMZM2G075588. Identical residues are highlighted in gray, and similar residues are highlighted in yellow and cyan.

(C) Developmental expression pattern of *ZmVAMP726*. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. *Zm00001d015327* (*UBQ*) was used as the reference. Error bars represent SD ($n = 3$).

(D) Quantification of H, G, and S units and total lignin monomers in thioacidolysis-released products of sporopollenin from B73 and *Zmvamp726*. Student's *t*-test was used for statistical analysis ($n = 10$, $P < 0.05$). Error bars represent SD. Asterisks indicate significant differences. n.s., not significant.

potential role during pollen development. To validate the effect of *ZmVAMP726* on lignin monomer composition in sporopollenin, we obtained two maize mutants (in the B73 background) carrying null mutations in *ZmVAMP726*. One of the mutants (named *Zmvamp726-1* and carrying a premature stop codon in the first exon) exhibited severe defects in pollen production owing to anther indehiscence (Supplemental Figure 9A). We therefore used the remaining mutant, which carried a premature stop codon in the third exon (named *Zmvamp726-2*, and hereafter *Zmvamp726*, Supplemental Figure 9A and 9B) for further analysis. We analyzed the ultrastructure of the pollen cell wall in wild-type maize B73 and *Zmvamp726* using scanning electron microscopy and transmission electron microscopy. The results revealed no visible changes in the surface or cell wall of pollen grains (Supplemental Figure 9C). However, the total content of lignin monomers and the abundance of H units in sporopollenin were significantly lower in *Zmvamp726* than in the B73 wild type, although levels of G and S lignin units were not altered (Figure 4D). Collectively, these results demonstrate that natural variation in *ZmVAMP726* is associated with lignin monomer composition in maize sporopollenin.

VAMP726 is involved in pollen resistance to heat and UV radiation in maize and *Arabidopsis*

The decreased content of total lignin monomers in sporopollenin of *Zmvamp726* suggested that its pollen grains may be hypersensitive to stresses such as heat and UV radiation. TTC staining revealed that a rather large number of pollen grains produced by *Zmvamp726* were nonviable in the absence of stress treatment

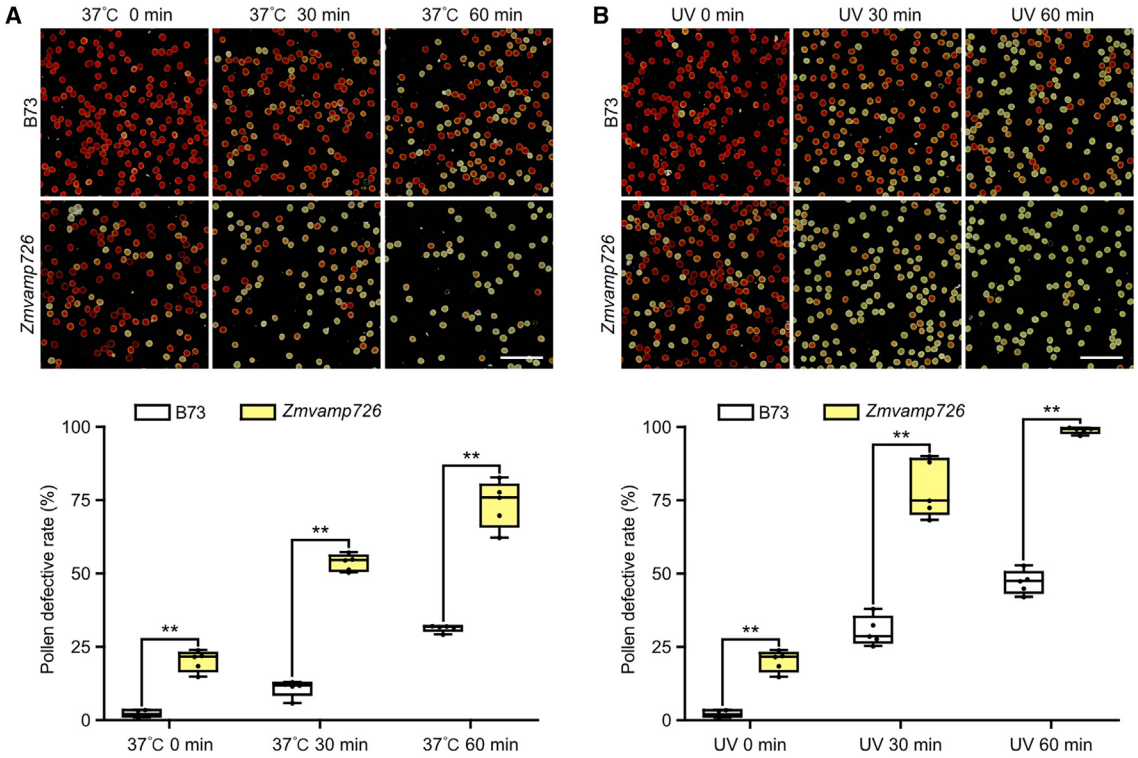

Figure 5. ZmVAMP726 is involved in maize pollen resistance to heat and UV radiation.

(A) Pollen viability of B73 and *Zmvamp726* after heat treatment. Fresh pollen grains were exposed to 37°C for the indicated times and then stained with TTC. Student's *t*-test was used for statistical analysis ($n = 5$, $P < 0.05$). Error bars represent SD. Asterisks indicate significant differences. Scale bar, 500 μm.

(B) Pollen viability of B73 and *Zmvamp726* after UV radiation treatment. Fresh pollen grains were exposed to UV for the indicated times and then stained with TTC. Student's *t*-test was used for statistical analysis ($n = 5$, $P < 0.05$). Error bars represent SD. Asterisks indicate significant differences. Scale bar, 500 μm.

(Figure 5A). The proportion of defective pollen grains increased with prolonged stress treatment in both B73 and *Zmvamp726* (Figure 5A). However, heat treatment resulted in a significantly higher rate of defective pollen grains in *Zmvamp726* than in B73 (Figure 5A). The percentage of defective pollen grains produced by *Zmvamp726* was ~75% at 60 min after treatment, whereas this percentage was ~30% in B73 (Figure 5A). Likewise, most pollen grains of *Zmvamp726* (>95%) completely lost viability after exposure to UV for 60 min, whereas more than half of the pollen grains from B73 remained viable (Figure 5B). These results suggest that ZmVAMP726 promotes pollen resistance to heat and UV radiation.

Phylogenetic analysis of VAMP7 family proteins revealed that ZmVAMP726 falls within the same clade as AtVAMP721, AtVAMP722, AtVAMP725, and AtVAMP726, as well as several other VAMP7s from *Glycine max*, *Solanum lycopersicum*, *Triticum aestivum*, *Oryza sativa*, and *Setaria viridis* (Supplemental Figure 10A), suggesting the functional conservation of VAMP726 across different plant species. A gene expression map of *Arabidopsis* development and β-glucuronidase (GUS) staining of an *AtVAMP726*$_{pro}$*:GUS* reporter line showed that *AtVAMP726* is mainly expressed in pollen (Supplemental Figure 11), indicating that, like *ZmVAMP726*, *AtVAMP726* may also be associated with pollen development. A previous report found that an AtVAMP726-GFP fusion protein was localized mainly on the plasma membrane and was likely associated with vesicle trafficking (Uemura et al., 2004). We co-expressed AtVAMP726 fused to mCherry and ZmVAMP726 fused to GFP in *Nicotiana benthamiana*. The fluorescent signals of the two fusion proteins clearly overlapped (Supplemental Figure 10B). The similar spatiotemporal expression patterns of *ZmVAMP726* and *AtVAMP726* suggest that they may have similar functions.

We obtained an *Arabidopsis* T-DNA insertion mutant (*Atvamp726-tdna*) and two null-mutant alleles created via the CRISPR–Cas9 system (*Atvamp726-gr1* and *Atvamp726-gr2*) (Supplemental Figure 12A). In addition, we created a *VAMP726*-overexpressing line in the Col-0 background (*35S*$_{pro}$*:AtVAMP726*, Supplemental Figure 12C). No obvious differences in reproductive growth were observed between these materials and Col-0 (Supplemental Figure 12D). However, pollen grains of the mutants were generally smaller than those of Col-0 and *35S*$_{pro}$*:AtVAMP726* and showed an increased incidence of shape distortion (Supplemental Figure 12E). In the absence of stress, pollen germination rate was slightly but not significantly reduced in *Atvamp726-tdna* compared with Col-0 and *35S*$_{pro}$*:AtVAMP726*, but pollen germination was more strongly reduced in *Atvamp726-gr1* and *Atvamp726-gr2* (Figure 6A). After heat treatment for 30 min and 60 min, pollen grains of all three mutants exhibited a significantly reduced germination rate, and the declines were much steeper for

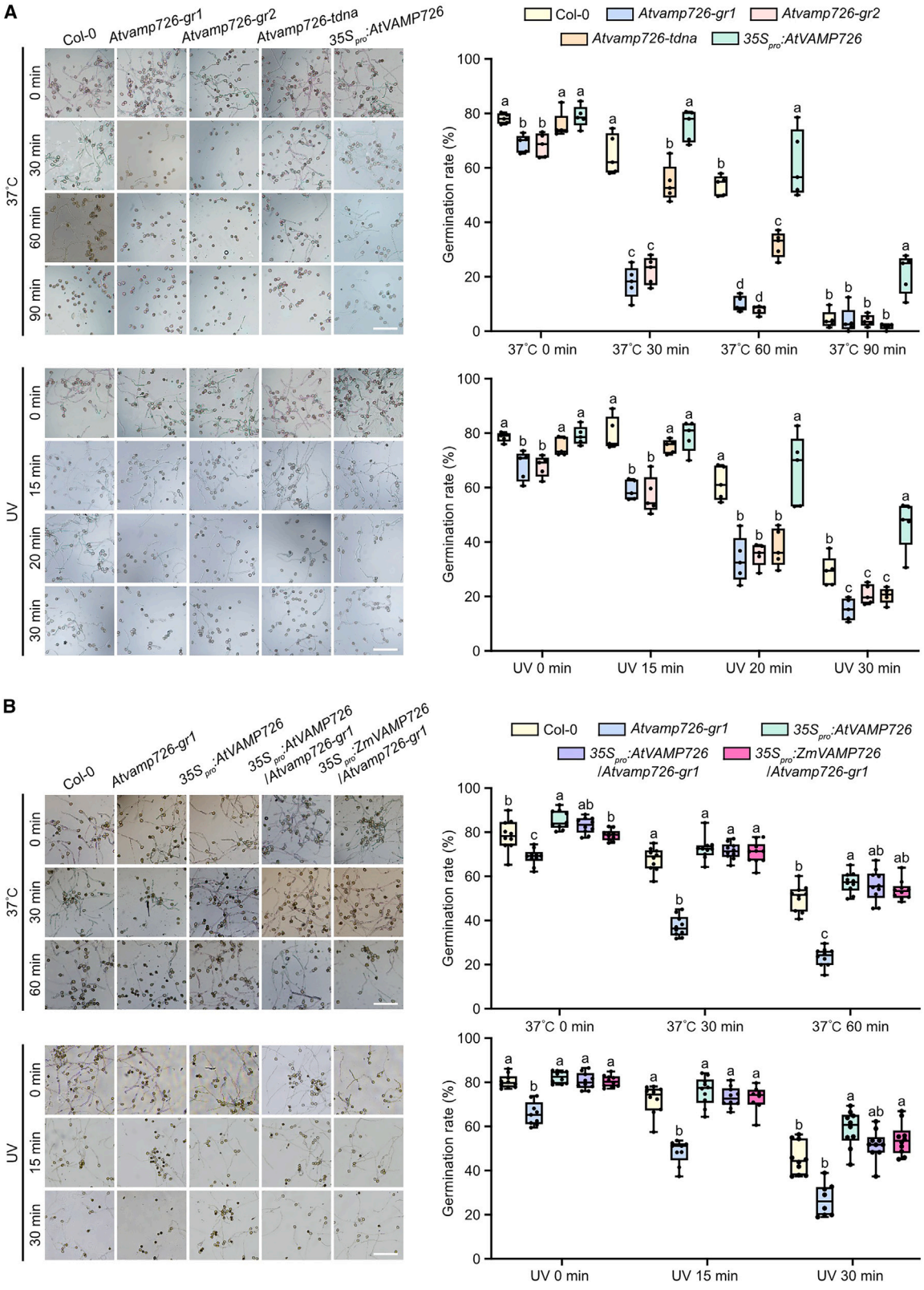

Figure 6. Both AtVAMP726 and ZmVAMP726 are involved in resistance of *Arabidopsis* pollen to heat and UV radiation.

(A) AtVAMP726 is involved in resistance of *Arabidopsis* pollen to heat and UV radiation. Fresh pollen grains of the indicated *Arabidopsis* genotypes were exposed to 37°C or UV radiation for the indicated times and then tested for *in vitro* germination. Two-way ANOVA followed by Tukey's honestly significant

(legend continued on next page)

Atvamp726-gr1 and *Atvamp726-gr2* (Figure 6A). Notably, pollen germination was less affected by heat treatment in $35S_{pro}$:*AtVAMP726* than in Col-0 (Figure 6A). Prolonged heat treatment (to 90 min) resulted in similar germination rates (less than 10%) of Col-0 and the three mutants, whereas over 20% of the $35S_{pro}$:*AtVAMP726* pollen grains remained viable (Figure 6A). Similar patterns in germination rate were observed when pollen grains were exposed to UV radiation (Figure 6A).

We next examined the lignin monomer composition in sporopollenin from the mutants and overexpression line. The abundance of H units and the total content of lignin monomers were significantly higher in sporopollenin of $35S_{pro:AtVAMP726}$ than in that of Col-0 and the two null mutants, but there were no significant differences in the abundance of G and S lignin units (Supplemental Figure 12F). However, in sporopollenin of *Atvamp726-gr1* and *Atvamp726-gr2*, we did detect a slight but not significant reduction in the abundance of each lignin monomer or the total content of lignin monomers (Supplemental Figure 12F). This might be attributed to the overall low level of lignin monomer accumulation in *Arabidopsis* sporopollenin. Nonetheless, these results suggest that AtVAMP726 mediates pollen resistance to heat and UV radiation, and genetic enhancement of *AtVAMP726* expression improved pollen resistance to these stresses, likely by influencing lignin monomer composition in sporopollenin.

We next wondered whether monocot and dicot VAMP726s could compensate for one another in the promotion of pollen stress resistance. We created transgenic *Arabidopsis* plants expressing *AtVAMP726* or *ZmVAMP726* under a 35S promoter in the *Atvamp726-gr1* background ($35S_{pro}$:*AtVAMP726*/*Atvamp726-gr1* and $35S_{pro}$:*ZmVAMP726*/*Atvamp726-gr1*, Supplemental Figure 13) and examined their pollen viability under heat and UV treatment. Under both stress conditions, constitutive expression of either *AtVAMP726* or *ZmVAMP726* in *Atvamp726-gr1* resulted in pollen grains that were more resistant to heat and UV treatment compared with Col-0 and *Atvamp726-gr1* (Figure 6B). Although the pollen resistance of $35S_{pro}$:*AtVAMP726*/*Atvamp726-gr1* and $35S_{pro}$:*ZmVAMP726*/*Atvamp726-gr1* was slightly lower than that of $35S_{pro}$:*AtVAMP726* (Col-0 background, Figure 6B), there was no difference in pollen viability between these two complemented lines, suggesting that heterologous expression of *ZmVAMP726* can fully restore pollen resistance of *Atvamp726-gr1*. Together, these results demonstrate the functional conservation of VAMP726 in maize and *Arabidopsis*.

Overexpression of *VAMP726* led to enrichment of peroxidases involved in lignification in the apoplastic space

We speculated that VAMP726 was a key determinant of lignin monomer composition in sporopollenin. As a protein involved in vesicle trafficking, VAMP726 likely mediates the transport of either metabolite units of sporopollenin or proteins involved in sporopollenin development from the cytosol to the apoplast. However, the cargo of VAMP726 may not necessarily be monolignols, because sporopollenin also contains long-chain polyvinyl alcohol units and coumaroylated aliphatic units (Li et al., 2019). Owing to technical limitations, we were not able to quantify these metabolite units in sporopollenin from maize or *Arabidopsis*. Nevertheless, the available facilities enabled us to determine which kinds of aliphatic units were present in thioacidolysis-released fractions of maize and *Arabidopsis* sporopollenin. We identified three fatty acids and three glycerides in the thioacidolysis-released products of maize sporopollenin (Supplemental Figure 14A). Semi-quantification revealed no differences in their abundance between sporopollenin from B73 and *Zmvamp726* (Supplemental Figure 14B). Similarly, there was no significant difference in the abundance of two fatty acids and two glycerides detected in the thioacidolysis-released products of sporopollenin from Col-0, *Atvamp726* mutants, and $35S_{pro}$:*AtVAMP726* (Supplemental Figure 14C). These results suggest that at least the abundance of aliphatic units released from sporopollenin by thioacidolysis is not associated with VAMP726.

Another member of the VAMP72 subgroup, VAMP721, has been shown to mediate translocation of cell-wall-modifying enzymes from the cytosol to the apoplastic space (Uemura et al., 2019). We speculated that VAMP726 may also transport proteins involved in the development of sporopollenin. To explore this possibility, we analyzed the proteome of apoplastic washing fluid (AWF) from mature leaves of *Arabidopsis* plants overexpressing *VAMP726* or an empty vector (Supplemental Datasets 4 and 5). To reduce costs, we did not use the *Atvamp726* mutant because of the low expression of *VAMP726* in its mature leaf tissues (Supplemental Figure 11). Proteomics analysis revealed that the abundance of 761 proteins in the apoplastic space was significantly altered by overexpression of *VAMP726* (Supplemental Dataset 6). Gene ontology (GO) enrichment analysis of these differentially accumulated proteins revealed significant enrichment of a number of biological processes, including "peroxidase activity" (Figure 7A and Supplemental Dataset 7), suggesting that overexpression of *VAMP726* may significantly affect this process. Eight peroxidases (PRXs) and three ascorbate peroxidases (APXs) were associated with this GO term (Supplemental Dataset 8). PRXs and laccases are two major classes of oxidative enzymes that catalyze the radical coupling of monolignols to form lignin (Tobimatsu and Schuetz, 2019). A recent study also showed that a mitochondrial APX is responsible for autonomous lignification at the earliest stage of xylem development (Zhang et al., 2022). Therefore, proteins associated with this GO term may be involved in the lignin polymerization process. Because PRXs and APXs include many members and not all of them are likely to be involved in lignin polymerization, we focused on the 11 proteins associated with this GO term and looked for published evidence supporting their potential role in lignification. PRX4 and PRX25 have

difference test was used for statistical analysis ($n = 5$, $P < 0.05$). Error bars represent SD. Letters indicate significant differences in germination rate at each time point. Scale bar, 200 μm.

(B) *ZmVAMP726* restored pollen stress resistance in *Atvamp726*. Fresh pollen grains were exposed to 37°C or UV radiation for the indicated times and then tested for *in vitro* germination. Two-way ANOVA followed by Tukey's honestly significant difference test was used for statistical analysis ($n = 5$, $P < 0.05$). Error bars represent SD. Letters indicate significant differences in germination rate at each time point. Scale bar, 200 μm.

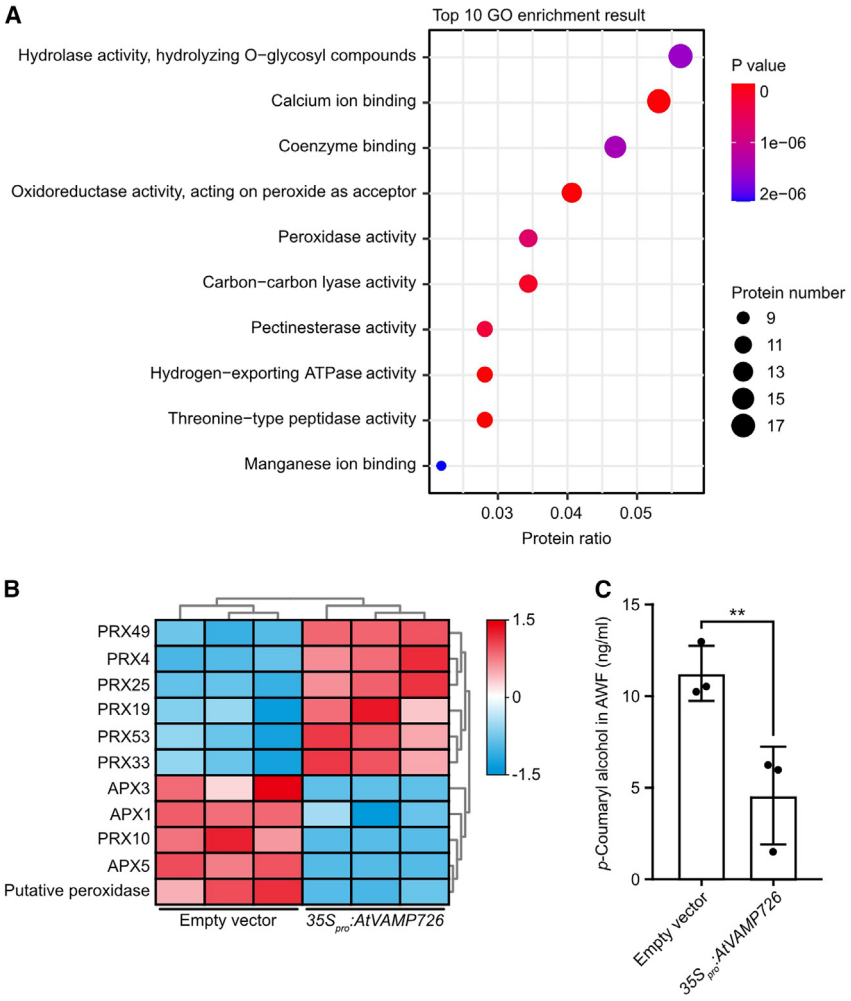

Figure 7. Overexpression of *AtVAMP726* alters the proteome of the apoplastic space.

(A) GO enrichment analysis of differentially accumulated proteins in apoplastic washing fluids obtained from *Arabidopsis* plants overexpressing *VAMP726*. The top ten most significantly enriched GO terms are listed.

(B) Relative abundance of proteins associated with the GO term "peroxidase activity." The protein abundances were normalized by *Z* score and plotted. Proteins were organized according to hierarchical clustering. Three biological replicates per genotype are represented by three columns. Color scale represents the *Z*-score value.

(C) Quantification of *p*-coumaryl alcohol in apoplastic washing fluids obtained from *Arabidopsis* plants. Student's *t*-test was used for statistical analysis ($n = 3$, $P < 0.05$). Error bars represent SD. Asterisks indicate a significant difference.

previously been shown to participate in lignin polymerization of *Arabidopsis* (Shigeto et al., 2013, 2014, 2015; Fernandez-Perez et al., 2015). Likewise, PRX49 and PRX53 have been proposed to function in lignin polymerization (Ostergaard et al., 2000; Herrero et al., 2013; Shigeto et al., 2014), although more experimental evidence is needed. These four PRXs were all among the proteins enriched in AWF of the *VAMP726*-overexpressing line (Figure 7B), suggesting that VAMP726 potentially mediates accumulation of these PRXs in the apoplastic space. No other PRXs annotated with this GO term have been reported or predicted to function in lignin polymerization in *Arabidopsis*. By contrast, the abundance of three APXs in the apoplastic space was negatively affected by overexpression of *VAMP726* (Figure 7B). APX1, also known as C3H, is involved in the early steps of monolignol biosynthesis in *Arabidopsis* (Barros et al., 2019). There is no evidence to support a role for APX1 in lignin polymerization in the apoplast. The other two APXs have not been reported or predicted to function in lignin-related processes in *Arabidopsis*. Together, these results suggest that VAMP726 is associated with the enrichment of PRXs involved in lignin polymerization in the apoplastic space.

These enriched PRXs might speed up the lignification process, resulting in more rapid consumption of monolignols in the apoplastic space. We therefore attempted to quantify the concentration of monolignols in the AWF. We were able to detect the presence of *p*-coumaryl alcohol in the AWF but were unable to detect the other two monolignols (Figure 7C). The results showed that the abundance of *p*-coumaryl alcohol in the AWF was significantly lower in $35S_{pro}$:*AtVAMP726* (Figure 7C), which is consistent with our speculation and with the increased lignin monomers detected in sporopollenin of $35S_{pro}$:*AtVAMP726* (Supplemental Figure 12F). Although the results presented above do not fully clarify the mechanism by which VAMP726 influences lignin monomer content in sporopollenin, they do suggest the possibility that VAMP726 may mediate translocation of enzymes involved in lignin polymerization to the apoplastic space.

DISCUSSION

The tough nature of sporopollenin makes it extremely resistant to physical and chemical degradation (Grienenberger and Quilichini, 2021). This property has hindered the elucidation of its chemical composition for decades. Two recent reports confirmed that aliphatic and phenolic compounds are structural components of sporopollenin in vascular plants (Li et al., 2019; Xue et al., 2020). Here, we provide compelling evidence that H, G, and S lignin units are also present in sporopollenin of maize and *Arabidopsis*. Interestingly, only G units were found previously in sporopollenin of *Cryptomeria* (gymnosperm) and *Lilium* (dicot) (Xue et al., 2020). This could be explained by the fact that the lignin monomer composition is species specific (Campbell and Sederoff, 1996). Nonetheless, our results and previous reports strongly support the notion that lignin monomers are structural components of plant sporopollenin.

Formation of the lignin polymer is considered to be a major event that enabled vascular plants to colonize terrestrial environments (Weng and Chapple, 2010). The lignin polymer can crosslink with hemicelluloses to form a specialized structure that permits

plants to transport water and stand upright (Terrett and Dupree, 2019). In addition, it is essential for helping plants to cope with diverse environmental stresses (Cesarino, 2019). For example, lignin can be deposited at infection sites of incompatible pathogens to form a physical barrier, thereby restricting their proliferation (Lee et al., 2019). Accordingly, disruption of monolignol biosynthesis in *Arabidopsis* drastically impairs disease resistance (Quentin et al., 2009; Tronchet et al., 2010). Likewise, monolignol biosynthesis is also indispensable for pollen resistance to excessive UV radiation (Xue et al., 2020). Here, we showed that deficiency in monolignol biosynthesis also impairs pollen resistance to heat stress, highlighting the essential role of monolignol biosynthesis in determining the outcome of plant–environment interactions. Although we were able to identify and quantify lignin monomers in sporopollenin, it remains unclear how they are incorporated into the main polymer structure. Thioacidolysis mainly breaks β-O-4 ether bonds between lignin monomers and ester bonds. Our data suggest that lignin monomers are not attached to other metabolite units of sporopollenin through ester bonds, indicating that they may form a lignin polymer in sporopollenin. However, they may also be linked to other metabolite units of sporopollenin through ether bonds. Novel analytical methods, such as solid-state NMR or energy-resolved mass spectrometry (Li et al., 2019; Dong et al., 2022), should be used to dissect the exact linkage patterns of lignin monomers with each other and with other sporopollenin constituents.

G and S lignin units are the dominant lignin monomers in the stem tissues of most plant species analyzed to date. H units usually comprise the minority of total lignin monomers in plants, with some exceptions in which the monolignol biosynthetic pathway was artificially modified (Franke et al., 2002; Li et al., 2010). Lignin monomer composition also exhibits tissue specificity. Lignin of water-conducting tracheids and vessels is particularly enriched in G units, whereas the proportion of S units increases significantly in lignin of fiber cells (Meyer et al., 1998; Nakashima et al., 2008). In Japanese cypress (*Chamaecyparis obtusa*, a gymnosperm), the proportion of H units is significantly higher in compression wood (22%) than in normal wood (1%) (Hiraide et al., 2021). Here, we found that H lignin comprised the majority of lignin monomers in sporopollenin of maize and *Arabidopsis*. This unique feature of sporopollenin is intriguing, as other parts of the plant rarely deposit such a high proportion of H units in the cell wall (which contains mainly G and S units). The composition of lignin monomers defines the physical and chemical characteristics of the lignin polymer. During lignin polymerization, the three lignin monomers are mostly interlinked via C–C and C–O linkages, and C–C linkages require higher dissociation energy than C–O linkages (Dong et al., 2019). Methoxy groups on the aromatic rings of lignin units can prevent the formation of interunit C–C linkages (Anderson et al., 2019). Therefore, the ratio of different lignin monomers can have a significant effect on the texture of the lignin polymer, and specific lignin chemotypes may be associated with specific functions (Renault et al., 2019). An increased proportion of H units would result in a highly condensed lignin structure, which is often associated with stress, as their relatively short biosynthetic pathway could provide readily accessible monolignols for rapid lignification to cope with stresses (Cesarino, 2019). If indeed the lignin monomers in sporopollenin form an integral polymer, it likely contributes to the superior durability of sporopollenin. In fact, sporopollenin can persist over millions of years, and the presence of relatively intact fossil sporopollenin in sediments provided the earliest evidence of land plants (Wellman et al., 2003). Previous work and our results demonstrate the key role of monolignol biosynthesis in pollen stress resistance (Xue et al., 2020). We therefore speculate that the unique enrichment of H units in sporopollenin might be an evolutionary adaptation that enables plants to cope with increased stresses, such as higher temperature and UV exposure, associated with colonization of the terrestrial environment. In this case, plants deposit more H units in sporopollenin to form a more condensed lignin polymer that protects genetic material from environmental stress.

We found that VAMP726 was positively involved in lignin monomer composition in sporopollenin and pollen resistance to heat and UV radiation. VAMPs, also known as R-type SNAREs, typically localize to membrane vesicles and drive membrane fusion with other organelles (Fujimoto et al., 2020). We made initial attempts to identify the cargo of VAMP726, and the results showed that overexpression of *VAMP726* resulted in enrichment of PRXs involved in lignin polymerization, supporting a possible role for VAMP726 in mediating the translocation of proteins involved in lignification. However, we also need to consider the possibility that VAMP726 is directly involved in transporting monolignols, as evidenced by its key role in the formation of autophagosomes in *Arabidopsis* (He et al., 2022). It was recently shown to deliver monolignols from the cytosol to the cell wall to facilitate rapid lignification upon pathogen attack (Jeon et al., 2023). Nonetheless, although previous reports have shown that transporter-based systems might mediate the translocation of monolignols (Miao and Liu, 2010; Alejandro et al., 2012), it remains debatable whether this mechanism is indispensable *in planta* (Perkins et al., 2019). A recent study demonstrated that monolignols could diffuse across the plasma membrane when a concentration gradient was present, and this process was unaffected by inhibition of transporter activity (Perkins et al., 2022). Therefore, the involvement of VAMP726 in monolignol transport might be conditional. VAMP726 is one of eight members of the VAMP72 subgroup (Zhang et al., 2015). Previous reports have demonstrated that two members of this group, VAMP721 and VAMP722, can form a SNARE complex with plasma-membrane-localized Qa-SNAREs to transport immune proteins, cell-wall-modification enzymes, secondary metabolites, and cell-wall components to the extracellular space (Kwon et al., 2008; Uemura et al., 2019; Yun et al., 2022). Therefore, it is likely that VAMP726 also interacts with other SNAREs to mediate translocation of metabolites or proteins required for accumulation of lignin monomers in sporopollenin. More comprehensive investigations are required to address the exact role of VAMP726 in the incorporation of lignin monomers into sporopollenin.

METHODS

Plant materials

The association mapping panel of maize materials was kindly provided by Dr. Jianbing Yan (Huazhong Agricultural

University) and consisted of 415 inbred lines (Yang et al., 2010). Among these, we were able to harvest sufficient pollen for sporopollenin analysis from 117 inbred lines. Hence, data obtained from 117 inbred lines were used for GWASs. *Zmvamp726-1* and *Zmvamp726-2* mutants carrying premature stop codons were obtained from an ethyl methanesulfonate mutant library of B73 (Lu et al., 2018). The premature stop codon in *Zmvamp726-1* is located in the first exon, and the premature stop codon in *Zmvamp726-2* is located in the third exon. Because *Zmvamp726-1* showed anther indehiscence, only *Zmvamp726-2* was backcrossed with B73, and its progeny were self-crossed for two generations (BC_1F_2). This BC_{1F_2} generation of *Zmvamp726-2* was used in subsequent work. For *Arabidopsis*, the Col-0 ecotype was preserved in the lab. T-DNA insertion mutants *tt4-3* (CS66119), *ccr1* (GK-622C01), *comt* (Salk_135290), *ccomt* (Salk_055103), and *vamp726* (Salk_082690) were obtained from Nottingham Arabidopsis Stock Center (Scholl et al., 2000). For simplicity, these mutants are mentioned without specific allele names in the main text. A detailed description of growth conditions is included in supplemental information.

Thioacidolysis of cell-wall residues

Pollen grains or other maize tissues were collected as described in supplemental information. Cell-wall residues were prepared according to a previously described protocol (Xue et al., 2020) with slight modifications. Thioacidolysis of cell-wall residues was performed as described previously (Foster et al., 2010). Detailed descriptions of these procedures are included in supplemental information.

Solution-state two-dimensional nuclear magnetic resonance

Approximately 2 g of cell-wall residue was treated with 300 ml of solution containing 1% Macerozyme R10 and 1% Cellulase Onozuka R10 (w/v, dissolved in 0.1 M sodium acetate buffer [pH 4.5]) at 37°C for 16 h. The resulting materials were washed with water and acetone three times and then subjected to thioacidolysis. For solution-state 2D-NMR spectroscopy, the thioacidolysis lysates of cell-wall residues were dissolved in 0.55 ml of deuterated DMSO-*d6* (99.9%, Sigma). A detailed description of this procedure is included in supplemental information.

Gas chromatography–mass spectrometry

For GC–MS, at least 2 mg of cell-wall residue was required for thioacidolysis, and the thioacidolysis lysate was then derivatized as described previously (Foster et al., 2010). A detailed description of this procedure is included in supplemental information.

Assessment of pollen viability

For heat treatment, fresh pollen grains of maize were placed in a 37°C incubator for the indicated times. For UV radiation, the pollen grains were placed under a UV lamp (6 W, 25 cm distance) for the indicated times. *Arabidopsis pollen grains* were spread onto solid basic medium before the stresses were applied. Pollen viability was then examined immediately. TTC staining was used to determine maize pollen viability (Alexander, 1969), and an *in vitro* germination assay was used to determine *Arabidopsis* pollen viability. Detailed descriptions of these procedures are included in supplemental information.

Genome-wide association study

The MLM model implemented in TASSEL 3.0 was used for GWAS (Bradbury et al., 2007; Li et al., 2013). Outliers were removed before the phenotype data were imported into TASSEL. The suggested P value of 2.04×10^{-6} (1/En) was used as the genome-wide threshold for significant SNP–trait associations, as is common in plant GWASs. The previously estimated decay distance of linkage disequilibrium (LD) in this association mapping panel (~30 kb, R^2 = 0.1) was used to define a 60-kb QTL interval, i.e., the 30 kb upstream and downstream of each SNP. The B73 reference genome (RefGen_v2) was used for annotation of candidate genes. Other details of the GWASs are included in supplemental information.

Proteome analysis of apoplastic washing fluid

AWF of *Arabidopsis* plants was obtained using the infiltration method as described previously (Chen et al., 2022). Proteins were extracted as described in supplemental information. The protein pellets were dissolved in 8 M urea, and the protein concentrations were determined using a detergent-compatible Bradford Protein Assay Kit (Beyotime Biotechnology). Label-free proteome analysis was performed as described previously (Yu et al., 2022).

Owing to space limitations, detailed descriptions of liquid chromatography coupled with triple-quadrupole mass spectrometry, scanning electron microscopy, collection of washing fractions during sporopollenin preparation, quantitative real-time PCR, GUS staining, phylogenetic analysis, and plant transformation are included in supplemental information.

DATA AND CODE AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

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AUTHOR CONTRIBUTIONS

Xuebin Zhang conceived and supervised the project. Xuebin Zhang, K.Y., W.Y., D.Y., and H.D. designed the experiments. W.Y., D.Y., and H.D. performed most of the experiments and analyzed the data. J.Z. established methods for detection of monolignols. Y.C. performed analysis of sporopollenin derived from *Arabidopsis* mutants. J.Z., Y.C., C.L., B.Z., and Y.M. assisted in experiments, including collection of maize materials in the field,

sample preparation, and GC–MS. Y.Z. performed initial sporopollenin analysis of maize inbred lines. E.Y. performed NMR spectroscopy. X.L., Xuehai Zhang, and J.T. provided materials and helped with experimental design and data analysis. W.Y., K.Y., and Xuebin Zhang prepared graphs and wrote the manuscript with input from all co-authors. All authors read and approved the manuscript.

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REFERENCES

Alejandro, S., Lee, Y., Tohge, T., Sudre, D., Osorio, S., Park, J., Bovet, L., Lee, Y., Geldner, N., Fernie, A.R., and Martinoia, E. (2012). AtABCG29 is a monolignol transporter involved in lignin biosynthesis. Curr. Biol. **22**:1207–1212. https://doi.org/10.1016/j.cub.2012.04.064.

Alexander, M.P. (1969). Differential staining of aborted and nonaborted pollen. Stain Technol. **44**:117–122. https://doi.org/10.3109/10520296909063335.

Anderson, E.M., Stone, M.L., Katahira, R., Reed, M., Muchero, W., Ramirez, K.J., Beckham, G.T., and Román-Leshkov, Y. (2019). Differences in S/G ratio in natural poplar variants do not predict catalytic depolymerization monomer yields. Nat. Commun. **10**:2033. https://doi.org/10.1038/s41467-019-09986-1.

Ariizumi, T., and Toriyama, K. (2011). Genetic regulation of sporopollenin synthesis and pollen exine development. Annu. Rev. Plant Biol. **62**:437–460. https://doi.org/10.1146/annurev-arplant-042809-112312.

Barros, J., Escamilla-Trevino, L., Song, L., Rao, X., Serrani-Yarce, J.C., Palacios, M.D., Engle, N., Choudhury, F.K., Tschaplinski, T.J., Venables, B.J., et al. (2019). 4-Coumarate 3-hydroxylase in the lignin biosynthesis pathway is a cytosolic ascorbate peroxidase. Nat. Commun. **10**:1994. https://doi.org/10.1038/s41467-019-10082-7.

Bonawitz, N.D., and Chapple, C. (2010). The genetics of lignin biosynthesis: connecting genotype to phenotype. Annu. Rev. Genet. **44**:337–363. https://doi.org/10.1146/annurev-genet-102209-163508.

Bradbury, P.J., Zhang, Z., Kroon, D.E., Casstevens, T.M., Ramdoss, Y., and Buckler, E.S. (2007). TASSEL: software for association mapping of complex traits in diverse samples. Bioinformatics **23**:2633–2635. https://doi.org/10.1093/bioinformatics/btm308.

Campbell, M.M., and Sederoff, R.R. (1996). Variation in lignin content and composition (mechanisms of control and implications for the genetic improvement of plants). Plant Physiol. **110**:3–13. https://doi.org/10.1104/pp.110.1.3.

Cesarino, I. (2019). Structural features and regulation of lignin deposited upon biotic and abiotic stresses. Curr. Opin. Biotechnol. **56**:209–214. https://doi.org/10.1016/j.copbio.2018.12.012.

Chaturvedi, P., Wiese, A.J., Ghatak, A., Záveská Drábková, L., Weckwerth, W., and Honys, D. (2021). Heat stress response mechanisms in pollen development. New Phytol. **231**:571–585. https://doi.org/10.1111/nph.17380.

Chen, A., He, B., and Jin, H. (2022). Isolation of extracellular vesicles from *Arabidopsis*. Curr. Protoc. **2**:e352. https://doi.org/10.1002/cpz1.352.

Choi, H., Jin, J.Y., Choi, S., Hwang, J.U., Kim, Y.Y., Suh, M.C., and Lee, Y. (2011). An ABCG/WBC-type ABC transporter is essential for transport of sporopollenin precursors for exine formation in developing pollen. Plant J. **65**:181–193. https://doi.org/10.1111/j.1365-313X.2010.04412.x.

Choi, H., Ohyama, K., Kim, Y.Y., Jin, J.Y., Lee, S.B., Yamaoka, Y., Muranaka, T., Suh, M.C., Fujioka, S., and Lee, Y. (2014). The role of *Arabidopsis* ABCG9 and ABCG31 ATP binding cassette transporters in pollen fitness and the deposition of steryl glycosides on the pollen coat. Plant Cell **26**:310–324. https://doi.org/10.1105/tpc.113.118935.

Del Río, J.C., Rencoret, J., Gutiérrez, A., Kim, H., and Ralph, J. (2018). Structural characterization of lignin from maize (*Zea mays* L.) fibers: evidence for diferuloylputrescine incorporated into the lignin polymer in maize kernels. J. Agric. Food Chem. **66**:4402–4413. https://doi.org/10.1021/acs.jafc.8b00880.

Dixon, R.A., and Paiva, N.L. (1995). Stress-Induced Phenylpropanoid Metabolism. Plant Cell **7**:1085–1097. https://doi.org/10.1105/tpc.7.7.1085.

Do, C.T., Pollet, B., Thévenin, J., Sibout, R., Denoue, D., Barrière, Y., Lapierre, C., and Jouanin, L. (2007). Both caffeoyl Coenzyme A 3-O-methyltransferase 1 and caffeic acid O-methyltransferase 1 are involved in redundant functions for lignin, flavonoids and sinapoyl malate biosynthesis in *Arabidopsis*. Planta **226**:1117–1129. https://doi.org/10.1007/s00425-007-0558-3.

Dolferus, R., Ji, X., and Richards, R.A. (2011). Abiotic stress and control of grain number in cereals. Plant Sci. **181**:331–341. https://doi.org/10.1016/j.plantsci.2011.05.015.

Dong, L., Lin, L., Han, X., Si, X., Liu, X., Guo, Y., Lu, F., Rudić, S., Parker, S.F., Yang, S., and Wang, Y. (2019). Breaking the limit of lignin monomer production via cleavage of interunit carbon–carbon linkages. Chem **5**:1521–1536. https://doi.org/10.1016/j.chempr.2019.03.007.

Dong, N.Q., and Lin, H.X. (2021). Contribution of phenylpropanoid metabolism to plant development and plant-environment interactions. J. Integr. Plant Biol. **63**:180–209. https://doi.org/10.1111/jipb.13054.

Dong, X., Mayes, H.B., Morreel, K., Katahira, R., Li, Y., Ralph, J., Black, B.A., and Beckham, G.T. (2022). Energy-Resolved Mass Spectrometry as a Tool for Identification of Lignin Depolymerization Products. ChemSusChem. https://doi.org/10.1002/cssc.202201441.

Edlund, A.F., Swanson, R., and Preuss, D. (2004). Pollen and stigma structure and function: the role of diversity in pollination. Plant Cell Suppl. **16**:S84–S97. https://doi.org/10.1105/tpc.015800.

Eudes, A., George, A., Mukerjee, P., Kim, J.S., Pollet, B., Benke, P.I., Yang, F., Mitra, P., Sun, L., Cetinkol, O.P., et al. (2012). Biosynthesis and incorporation of side-chain-truncated lignin monomers to reduce lignin polymerization and enhance saccharification. Plant Biotechnol. J. **10**:609–620. https://doi.org/10.1111/j.1467-7652.2012.00692.x.

Fernández-Pérez, F., Vivar, T., Pomar, F., Pedreño, M.A., and Novo-Uzal, E. (2015). Peroxidase 4 is involved in syringyl lignin formation in *Arabidopsis thaliana*. J. Plant Physiol. **175**:86–94. https://doi.org/10.1016/j.jplph.2014.11.006.

Foster, C.E., Martin, T.M., and Pauly, M. (2010). Comprehensive compositional analysis of plant cell walls (Lignocellulosic biomass) part I: lignin. J. Vis. Exp. https://doi.org/10.3791/1745.

Franke, R., Hemm, M.R., Denault, J.W., Ruegger, M.O., Humphreys, J.M., and Chapple, C. (2002). Changes in secondary metabolism and deposition of an unusual lignin in the *ref8* mutant of *Arabidopsis*. Plant J. **30**:47–59. https://doi.org/10.1046/j.1365-313x.2002.01267.x.

Fraser, C.M., and Chapple, C. (2011). The phenylpropanoid pathway in *Arabidopsis*. *Arabidopsis* Book **9**, e0152. https://doi.org/10.1199/tab.0152.

Fujimoto, M., Ebine, K., Nishimura, K., Tsutsumi, N., and Ueda, T. (2020). Longin R-SNARE is retrieved from the plasma membrane by ANTH domain-containing proteins in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **117**:25150–25158. https://doi.org/10.1073/pnas.2011152117.

Grienenberger, E., and Quilichini, T.D. (2021). The toughest material in the plant kingdom: an update on sporopollenin. Front. Plant Sci. **12**, 703864. https://doi.org/10.3389/fpls.2021.703864.

He, Y., Gao, J., Luo, M., Gao, C., Lin, Y., Wong, H.Y., Cui, Y., Zhuang, X., and Jiang, L. (2022). VAMP724 and VAMP726 are involved in autophagosome formation in *Arabidopsis thaliana*. Autophagy **19**:1406–1423. https://doi.org/10.1080/15548627.2022.2127240.

Herrero, J., Esteban-Carrasco, A., and Zapata, J.M. (2013). Looking for *Arabidopsis thaliana* peroxidases involved in lignin biosynthesis. Plant Physiol. Biochem. **67**:77–86. https://doi.org/10.1016/j.plaphy.2013.02.019.

Hiraide, H., Tobimatsu, Y., Yoshinaga, A., Lam, P.Y., Kobayashi, M., Matsushita, Y., Fukushima, K., and Takabe, K. (2021). Localised laccase activity modulates distribution of lignin polymers in gymnosperm compression wood. New Phytol. **230**:2186–2199. https://doi.org/10.1111/nph.17264.

Hoopes, G.M., Hamilton, J.P., Wood, J.C., Esteban, E., Pasha, A., Vaillancourt, B., Provart, N.J., and Buell, C.R. (2019). An updated gene atlas for maize reveals organ-specific and stress-induced genes. Plant J. **97**:1154–1167. https://doi.org/10.1111/tpj.14184.

Huang, M.D., Chen, T.L.L., and Huang, A.H.C. (2013). Abundant type III lipid transfer proteins in *Arabidopsis* tapetum are secreted to the locule and become a constituent of the pollen exine. Plant Physiol. **163**:1218–1229. https://doi.org/10.1104/pp.113.225706.

Ichino, T., and Yazaki, K. (2022). Modes of secretion of plant lipophilic metabolites via ABCG transporter-dependent transport and vesicle-mediated trafficking. Curr. Opin. Plant Biol. **66**, 102184. https://doi.org/10.1016/j.pbi.2022.102184.

Jeon, H.S., Jang, E., Kim, J., Kim, S.H., Lee, M.H., Nam, M.H., Tobimatsu, Y., and Park, O.K. (2023). Pathogen-induced autophagy regulates monolignol transport and lignin formation in plant immunity. Autophagy **19**:597–615. https://doi.org/10.1080/15548627.2022.2085496.

Jiang, J., Zhang, Z., and Cao, J. (2013). Pollen wall development: the associated enzymes and metabolic pathways. Plant Biol. **15**:249–263. https://doi.org/10.1111/j.1438-8677.2012.00706.x.

Kim, H., Padmakshan, D., Li, Y., Rencoret, J., Hatfield, R.D., and Ralph, J. (2017). Characterization and elimination of undesirable protein residues in plant cell wall materials for enhancing lignin analysis by solution-state nuclear magnetic resonance spectroscopy. Biomacromolecules **18**:4184–4195. https://doi.org/10.1021/acs.biomac.7b01223.

Kwon, C., Neu, C., Pajonk, S., Yun, H.S., Lipka, U., Humphry, M., Bau, S., Straus, M., Kwaaitaal, M., Rampelt, H., et al. (2008). Co-option of a default secretory pathway for plant immune responses. Nature **451**:835–840. https://doi.org/10.1038/nature06545.

Lan, W., Lu, F., Regner, M., Zhu, Y., Rencoret, J., Ralph, S.A., Zakai, U.I., Morreel, K., Boerjan, W., and Ralph, J. (2015). Tricin, a flavonoid monomer in monocot lignification. Plant Physiol. **167**:1284–1295. https://doi.org/10.1104/pp.114.253757.

Lee, M.H., Jeon, H.S., Kim, S.H., Chung, J.H., Roppolo, D., Lee, H.J., Cho, H.J., Tobimatsu, Y., Ralph, J., and Park, O.K. (2019). Lignin-based barrier restricts pathogens to the infection site and confers resistance in plants. EMBO J. **38**, e101948. https://doi.org/10.15252/embj.2019101948.

Li, F.S., Phyo, P., Jacobowitz, J., Hong, M., and Weng, J.K. (2019). The molecular structure of plant sporopollenin. Nat. Plants **5**:41–46. https://doi.org/10.1038/s41477-018-0330-7.

Li, H., Peng, Z., Yang, X., Wang, W., Fu, J., Wang, J., Han, Y., Chai, Y., Guo, T., Yang, N., et al. (2013). Genome-wide association study dissects the genetic architecture of oil biosynthesis in maize kernels. Nat. Genet. **45**:43–50. https://doi.org/10.1038/ng.2484.

Li, X., Bonawitz, N.D., Weng, J.K., and Chapple, C. (2010). The growth reduction associated with repressed lignin biosynthesis in *Arabidopsis thaliana* is independent of flavonoids. Plant Cell **22**:1620–1632. https://doi.org/10.1105/tpc.110.074161.

Lu, X., Liu, J., Ren, W., Yang, Q., Chai, Z., Chen, R., Wang, L., Zhao, J., Lang, Z., Wang, H., et al. (2018). Gene-indexed mutations in maize. Mol. Plant **11**:496–504. https://doi.org/10.1016/j.molp.2017.11.013.

Meyer, K., Shirley, A.M., Cusumano, J.C., Bell-Lelong, D.A., and Chapple, C. (1998). Lignin monomer composition is determined by the expression of a cytochrome P450-dependent monooxygenase in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **95**:6619–6623. https://doi.org/10.1073/pnas.95.12.6619.

Miao, Y.C., and Liu, C.J. (2010). ATP-binding cassette-like transporters are involved in the transport of lignin precursors across plasma and vacuolar membranes. Proc. Natl. Acad. Sci. USA **107**:22728–22733. https://doi.org/10.1073/pnas.1007747108.

Nakashima, J., Chen, F., Jackson, L., Shadle, G., and Dixon, R.A. (2008). Multi-site genetic modification of monolignol biosynthesis in alfalfa (*Medicago sativa*): effects on lignin composition in specific cell types. New Phytol. **179**:738–750. https://doi.org/10.1111/j.1469-8137.2008.02502.x.

Ostergaard, L., Teilum, K., Mirza, O., Mattsson, O., Petersen, M., Welinder, K.G., Mundy, J., Gajhede, M., and Henriksen, A. (2000). *Arabidopsis* ATP A2 peroxidase. Expression and high-resolution structure of a plant peroxidase with implications for lignification. Plant Mol. Biol. **44**:231–243. https://doi.org/10.1023/a:1006442618860.

Perkins, M., Smith, R.A., and Samuels, L. (2019). The transport of monomers during lignification in plants: anything goes but how? Curr. Opin. Biotechnol. **56**:69–74. https://doi.org/10.1016/j.copbio.2018.09.011.

Perkins, M.L., Schuetz, M., Unda, F., Chen, K.T., Bally, M.B., Kulkarni, J.A., Yan, Y., Pico, J., Castellarin, S.D., Mansfield, S.D., and Samuels, A.L. (2022). Monolignol export by diffusion down a polymerization-induced concentration gradient. Plant Cell **34**:2080–2095. https://doi.org/10.1093/plcell/koac051.

Qian, Y., Qiu, X., and Zhu, S. (2015). Lignin: a nature-inspired sun blocker for broad-spectrum sunscreens. Green Chem. **17**:320–324. https://doi.org/10.1039/c4gc01333f.

Quentin, M., Allasia, V., Pegard, A., Allais, F., Ducrot, P.H., Favery, B., Levis, C., Martinet, S., Masur, C., Ponchet, M., et al. (2009). Imbalanced lignin biosynthesis promotes the sexual reproduction of homothallic oomycete pathogens. PLoS Pathog. **5**, e1000264. https://doi.org/10.1371/journal.ppat.1000264.

Quilichini, T.D., Samuels, A.L., and Douglas, C.J. (2014). ABCG26-mediated polyketide trafficking and hydroxycinnamoyl spermidines contribute to pollen wall exine formation in *Arabidopsis*. Plant Cell **26**:4483–4498. https://doi.org/10.1105/tpc.114.130484.

Ralph, J., Lapierre, C., and Boerjan, W. (2019). Lignin structure and its engineering. Curr. Opin. Biotechnol. **56**:240–249. https://doi.org/10.1016/j.copbio.2019.02.019.

Renault, H., Werck-Reichhart, D., and Weng, J.K. (2019). Harnessing lignin evolution for biotechnological applications. Curr. Opin. Biotechnol. **56**:105–111. https://doi.org/10.1016/j.copbio.2018.10.011.

Rinaldi, R., Jastrzebski, R., Clough, M.T., Ralph, J., Kennema, M., Bruijnincx, P.C.A., and Weckhuysen, B.M. (2016). Paving the way

for lignin valorisation: recent advances in bioengineering, biorefining and catalysis. Angew. Chem., Int. Ed. Engl. **55**:8164–8215. https://doi.org/10.1002/anie.201510351.

Sanderfoot, A.A., Assaad, F.F., and Raikhel, N.V. (2000). The *Arabidopsis* genome. An abundance of soluble *N*-ethylmaleimide-sensitive factor adaptor protein receptors. Plant Physiol. **124**:1558–1569. https://doi.org/10.1104/pp.124.4.1558.

Scholl, R.L., May, S.T., and Ware, D.H. (2000). Seed and molecular resources for *Arabidopsis*. Plant Physiol. **124**:1477–1480. https://doi.org/10.1104/pp.124.4.1477.

Shi, J., Cui, M., Yang, L., Kim, Y.J., and Zhang, D. (2015). Genetic and biochemical mechanisms of pollen wall development. Trends Plant Sci. **20**:741–753. https://doi.org/10.1016/j.tplants.2015.07.010.

Shigeto, J., Nagano, M., Fujita, K., and Tsutsumi, Y. (2014). Catalytic profile of *Arabidopsis* peroxidases, AtPrx-2, 25 and 71, contributing to stem lignification. PLoS One **9**, e105332. https://doi.org/10.1371/journal.pone.0105332.

Shigeto, J., Kiyonaga, Y., Fujita, K., Kondo, R., and Tsutsumi, Y. (2013). Putative cationic cell-wall-bound peroxidase homologues in *Arabidopsis*, *AtPrx2*, *AtPrx25*, and *AtPrx71*, are involved in lignification. J. Agric. Food Chem. **61**:3781–3788. https://doi.org/10.1021/jf400426g.

Shigeto, J., Itoh, Y., Hirao, S., Ohira, K., Fujita, K., and Tsutsumi, Y. (2015). Simultaneously disrupting *AtPrx2*, *AtPrx25* and *AtPrx71* alters lignin content and structure in *Arabidopsis* stem. J. Integr. Plant Biol. **57**:349–356. https://doi.org/10.1111/jipb.12334.

Stapleton, A.E. (1992). Ultraviolet radiation and plants: burning questions. Plant Cell **4**:1353–1358. https://doi.org/10.1105/tpc.4.11.1353.

Sun, Q., Liu, X., Yang, J., Liu, W., Du, Q., Wang, H., Fu, C., and Li, W.X. (2018). MicroRNA528 affects lodging resistance of maize by regulating lignin biosynthesis under nitrogen-luxury conditions. Mol. Plant **11**:806–814. https://doi.org/10.1016/j.molp.2018.03.013.

Terrett, O.M., and Dupree, P. (2019). Covalent interactions between lignin and hemicelluloses in plant secondary cell walls. Curr. Opin. Biotechnol. **56**:97–104. https://doi.org/10.1016/j.copbio.2018.10.010.

Tobimatsu, Y., and Schuetz, M. (2019). Lignin polymerization: how do plants manage the chemistry so well? Curr. Opin. Biotechnol. **56**:75–81. https://doi.org/10.1016/j.copbio.2018.10.001.

Tronchet, M., Balagué, C., Kroj, T., Jouanin, L., and Roby, D. (2010). Cinnamyl alcohol dehydrogenases-C and D, key enzymes in lignin biosynthesis, play an essential role in disease resistance in *Arabidopsis*. Mol. Plant Pathol. **11**:83–92. https://doi.org/10.1111/j.1364-3703.2009.00578.x.

Uemura, T., Ueda, T., Ohniwa, R.L., Nakano, A., Takeyasu, K., and Sato, M.H. (2004). Systematic analysis of SNARE molecules in *Arabidopsis*: dissection of the post-Golgi network in plant cells. Cell Struct. Funct. **29**:49–65. https://doi.org/10.1247/csf.29.49.

Uemura, T., Nakano, R.T., Takagi, J., Wang, Y., Kramer, K., Finkemeier, I., Nakagami, H., Tsuda, K., Ueda, T., Schulze-Lefert, P., and Nakano, A. (2019). A Golgi-released subpopulation of the trans-Golgi network mediates protein secretion in *Arabidopsis*. Plant Physiol. **179**:519–532. https://doi.org/10.1104/pp.18.01228.

Wang, K., Guo, Z.L., Zhou, W.T., Zhang, C., Zhang, Z.Y., Lou, Y., Xiong, S.X., Yao, X.Z., Fan, J.J., Zhu, J., and Yang, Z.N. (2018). The regulation of sporopollenin biosynthesis genes for rapid pollen wall formation. Plant Physiol. **178**:283–294. https://doi.org/10.1104/pp.18.00219.

Wellman, C.H., Osterloff, P.L., and Mohiuddin, U. (2003). Fragments of the earliest land plants. Nature **425**:282–285. https://doi.org/10.1038/nature01884.

Weng, J.K., and Chapple, C. (2010). The origin and evolution of lignin biosynthesis. New Phytol. **187**:273–285. https://doi.org/10.1111/j.1469-8137.2010.03327.x.

Wheeler, T., and von Braun, J. (2013). Climate change impacts on global food security. Science **341**:508–513. https://doi.org/10.1126/science.1239402.

Xue, J.S., Zhang, B., Zhan, H., Lv, Y.L., Jia, X.L., Wang, T., Yang, N.Y., Lou, Y.X., Zhang, Z.B., Hu, W.J., et al. (2020). Phenylpropanoid derivatives are essential components of sporopollenin in vascular plants. Mol. Plant **13**:1644–1653. https://doi.org/10.1016/j.molp.2020.08.005.

Yang, X., Gao, S., Xu, S., Zhang, Z., Prasanna, B.M., Li, L., Li, J., and Yan, J. (2010). Characterization of a global germplasm collection and its potential utilization for analysis of complex quantitative traits in maize. Mol. Breed. **28**:511–526. https://doi.org/10.1007/s11032-010-9500-7.

Yu, K., Yang, W., Zhao, B., Wang, L., Zhang, P., Ouyang, Y., Chang, Y., Chen, G., Zhang, J., Wang, S., et al. (2022). The Kelch-F-box protein SMALL AND GLOSSY LEAVES 1 (SAGL1) negatively influences salicylic acid biosynthesis in *Arabidopsis thaliana* by promoting the turn-over of transcription factor SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1 (SARD1). New Phytol. **235**:885–897. https://doi.org/10.1111/nph.18197.

Yue, F., Lu, F., Sun, R.C., and Ralph, J. (2012). Syntheses of lignin-derived thioacidolysis monomers and their uses as quantitation standards. J. Agric. Food Chem. **60**:922–928. https://doi.org/10.1021/jf204481x.

Yun, H.S., Sul, W.J., Chung, H.S., Lee, J.H., and Kwon, C. (2022). Secretory membrane traffic in plant-microbe interactions. New Phytol. **237**:53–59. https://doi.org/10.1111/nph.18470.

Zhang, B., Karnik, R., Wang, Y., Wallmeroth, N., Blatt, M.R., and Grefen, C. (2015). The *Arabidopsis* R-SNARE VAMP721 interacts with KAT1 and KC1 K^+ channels to moderate K+ current at the plasma membrane. Plant Cell **27**:1697–1717. https://doi.org/10.1105/tpc.15.00305.

Zhang, D., Liang, W., Yin, C., Zong, J., Gu, F., and Zhang, D. (2010). OsC6, encoding a lipid transfer protein, is required for postmeiotic anther development in rice. Plant Physiol. **154**:149–162. https://doi.org/10.1104/pp.110.158865.

Zhang, J., Liu, Y., Li, C., Yin, B., Liu, X., Guo, X., Zhang, C., Liu, D., Hwang, I., Li, H., and Lu, H. (2022). PtomtAPX is an autonomous lignification peroxidase during the earliest stage of secondary wall formation in Populus tomentosa Carr. Nat. Plants **8**:828–839. https://doi.org/10.1038/s41477-022-01181-3.