FIBRILLIN4 Is Required for Plastoglobule Development and Stress Resistance in Apple and Arabidopsis[1][W][OA]

Dharmendra K. Singh, Siela N. Maximova, Philip J. Jensen, Brian L. Lehman, Henry K. Ngugi, and Timothy W. McNellis*

Intercollege Graduate Degree Program in Plant Biology (D.K.S.), Department of Plant Pathology (D.K.S., P.J.J., H.K.N., T.W.M.), and Department of Horticulture (S.N.M.), The Pennsylvania State University, University Park, Pennsylvania 16802; and The Pennsylvania State University Fruit Research and Extension Center, Biglerville, Pennsylvania 17307 (B.L.L., H.K.N.)

The fibrillins are a large family of chloroplast proteins that have been linked with stress tolerance and disease resistance. FIBRILLIN4 (FIB4) is found associated with the photosystem II light-harvesting complex, thylakoids, and plastoglobules, which are chloroplast compartments rich in lipophilic antioxidants. For this study, *FIB4* expression was knocked down in apple (*Malus* × *domestica*) using RNA interference. Plastoglobule osmiophilicity was decreased in *fib4* knockdown (*fib4* KD) tree chloroplasts compared with the wild type, while total plastoglobule number was unchanged. Compared with the wild type, net photosynthetic CO_2 fixation in *fib4* KD trees was decreased at high light intensity but was increased at low light intensity. Furthermore, *fib4* KD trees produced more anthocyanins than the wild type when transferred from low to high light intensity, indicating greater sensitivity to high light stress. Relative to the wild type, *fib4* KD apples were more sensitive to methyl viologen and had higher superoxide levels during methyl viologen treatment. Arabidopsis (*Arabidopsis thaliana*) *fib4* mutants and *fib4* KD apples were more susceptible than their wild-type counterparts to the bacterial pathogens *Pseudomonas syringae* pathovar *tomato* and *Erwinia amylovora*, respectively, and were more sensitive to ozone-induced tissue damage. Following ozone stress, plastoglobule osmiophilicity decreased in wild-type apple and remained low in *fib4* KD trees; total plastoglobule number increased in *fib4* KD apples but not in the wild type. These results indicate that FIB4 is required for plastoglobule development and resistance to multiple stresses. This study suggests that FIB4 is involved in regulating plastoglobule content and that defective regulation of plastoglobule content leads to broad stress sensitivity and altered photosynthetic activity.

Increased production of reactive oxygen species (ROS) is among the first biochemical responses of plants when challenged by pathogens and harsh environmental conditions (Mehdy, 1994; Lamb and Dixon, 1997; Joo et al., 2005). ROS are implicated in tissue damage during environmental stress and in the promotion of disease development by necrotrophic and hemibiotrophic pathogens (Venisse et al., 2001; Apel and Hirt, 2004; Shetty et al., 2008). For example, ROS production is critical for host colonization and pathogenesis by the bacterium *Erwinia amylovora*, which causes fire blight disease in rosaceous plants such as apple (*Malus* × *domestica*) and pear (*Pyrus communis*; Venisse et al., 2001).

The chloroplast is a site of ROS production during biotic and abiotic stress (Joo et al., 2005; Liu et al., 2007). The chloroplast has a battery of enzymes such as superoxide dismutase and ascorbate peroxidase, and antioxidants such as ascorbate, glutathione, and tocopherols, for protection against ROS (Noctor and Foyer, 1998; Asada, 2006). Plastoglobules are lipoprotein bodies attached to the thylakoids (Austin et al., 2006) that store lipids, including antioxidants such as tocopherols, carotenes, and plastoquinones (Steinmüller and Tevini, 1985; Tevini and Steinmüller, 1985). In addition to antioxidants, plastoglobules contain tocopherol cyclase, which is involved in γ-tocopherol synthesis (Austin et al., 2006; Vidi et al., 2006). The antioxidant content of plastoglobules and their apparent involvement in tocopherol biosynthesis imply that they could play a role in plant responses to oxidative stress.

Plastoglobules contain fibrillins, which were initially described as protein components of chromoplast fibrils with a molecular mass of approximately 30 kD (Winkenbach et al., 1976; Knoth et al., 1986; Emter et al., 1990; Deruère et al., 1994). Fibrillins are ubiquitous proteins present from cyanobacteria to plants (Laizet et al., 2004). Fibrillins maintain plastoglobule structural integrity (Deruère et al., 1994; Pozueta-Romero et al., 1997; Langenkämper et al., 2001; Vidi et al., 2006; Bréhélin et al., 2007) and stabilize the photosynthetic apparatus during photooxidative stress (Gillet et al., 1998; Yang et al., 2006; Youssef

[1] This work was supported by the U.S. National Science Foundation Plant Genome Research Program (grant no. DBI–0420394 to T.W.M., Robert M. Crassweller, James W. Travis, and S.N.M.).

* Corresponding author; e-mail twm4@psu.edu.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Timothy W. McNellis (twm4@psu.edu).

[W] The online version of this article contains Web-only data.

[OA] Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.110.164095

et al., 2010), osmotic stress (Gillet et al., 1998), drought (Pruvot et al., 1996; Rey et al., 2000), and low temperature (Rorat et al., 2001). Fibrillins are involved in abscisic acid-mediated protection from photoinhibition (Yang et al., 2006), and a subfamily of Arabidopsis (*Arabidopsis thaliana*) fibrillins (FIB1a, -1b, and -2) conditions jasmonate production during low-temperature, photooxidative stress (Youssef et al., 2010). Arabidopsis plants lacking one fibrillin (At4g22240) and tomato (*Solanum lycopersicum*) plants with suppressed expression of a fibrillin (*LeCHRC*) are susceptible to *Pseudomonas syringae* and *Botrytis cinerea*, respectively (Cooper et al., 2003; Leitner-Dagan et al., 2006), indicating that fibrillins play a role in disease resistance.

The Arabidopsis fibrillin encoded by At3g23400 has received various appellations, including FIBRILLIN4 (FIB4; Laizet et al., 2004), Harpin-Binding Protein1 (Song et al., 2002), AtPGL 30.4 (Vidi et al., 2006), and Fibrillin6 (Galetskiy et al., 2008); here, it will be referred to by its earliest published name, FIB4. FIB4 is found associated with the PSII light-harvesting complex (Galetskiy et al., 2008). FIB4 has also been detected in plastoglobules (Vidi et al., 2006; Ytterberg et al., 2006) and thylakoids (Friso et al., 2004; Peltier et al., 2004). However, the specific function of FIB4 is unknown. Several lines of evidence suggest that FIB4 may be involved in plant disease resistance responses: pathogen-associated molecular patterns trigger its phosphorylation (Jones et al., 2006); pathogen-associated molecular patterns stimulate the expression of its ortholog in tobacco (*Nicotiana tabacum*; Jones et al., 2006; Sanabria and Dubery, 2006); and it can physically interact with the HrpN (harpin) virulence protein of the fire blight pathogen *E. amylovora* in a yeast two-hybrid assay, suggesting that it could be a receptor or target of HrpN (Song et al., 2002). In addition, it is thought that FIB4 may be involved in the transport of small, hydrophobic molecules because it contains a conserved lipocalin signature (Jones et al., 2006). Here, we report a genetic analysis of FIB4 function in apple and Arabidopsis in terms of its role in plastoglobule development and plant resistance to biotic and abiotic stresses.

RESULTS

Knockdown of *FIB4* Expression in Apple

We generated *fib4* knockdown (*fib4* KD) apple trees using the RNA interference (RNAi) technique in the cv Royal Gala (Supplemental Figs. S1 and S2A; Maximova et al., 1998). Transgenic, vector-only control (VC) plants carrying the transformation marker genes *Neomycin Phosphotransferase II* and *Enhanced Green Fluorescent Protein* (*EGFP*), but not the *FIB4* RNAi cassette, were also generated (Supplemental Figs. S1 and S2A). The level of *FIB4* transcript in *fib4* KD plantlets was less than 10% of the wild-type level, indicating successful silencing (Supplemental Fig. S2B). Wild-type, VC, and *fib4* KD apple trees were phenotypically indistinguishable when grown under normal laboratory conditions (Supplemental Fig. S2C).

Plastoglobule Ultrastructure

Since FIB4 is a component of the plastoglobule and is associated with the PSII light-harvesting complex, we investigated the effect of knockdown of *FIB4* expression on chloroplast and plastoglobule ultrastructure in apple. Leaf sections from *fib4* KD and wild-type apple trees were stained with osmium tetroxide (OsO_4) and observed by transmission electron microscopy (TEM). Overall chloroplast morphology appeared normal in *fib4* KD palisade mesophyll cells (Fig. 1A). Strikingly, however, *fib4* KD chloroplasts contained almost exclusively electron-transparent (nonosmiophilic, or white) plastoglobules, while wild-type chloroplasts contained many electron-opaque (osmiophilic, or black) plastoglobules (Fig. 1, A and B). Plastoglobules are thought to be osmiophilic because the unsaturated lipids they contain reduce OsO_4 (Padham et al., 2007). There were more white plastoglobules in *fib4* KD chloroplasts than wild-type chloroplasts, although the difference was not statistically significant (Fig. 1B). The total number of plastoglobules (electron transparent + electron opaque) was similar in *fib4* KD and wild-type chloroplasts (Fig. 1C). Peripheral vesicles were similar in number in *fib4* KD and wild-type chloroplasts (Fig. 1D). The peripheral vesicles were electron transparent, irregularly shaped, located adjacent to the chloroplast inner envelope membrane, and clearly distinguishable from the round, white plastoglobules (Fig. 1E). Black plastoglobules had varying patterns of electron opacity ranging from partially to fully electron opaque (Fig. 1, A, F, and G). Black and white plastoglobules were often observed in the same chloroplast, sometimes in juxtaposition (Fig. 1H).

Photosynthetic CO_2 Fixation

Since *fib4* KD apple tree plastoglobules exhibited altered ultrastructure, we decided to determine whether knockdown of *FIB4* affected photosynthesis rates. Net photosynthetic CO_2 assimilation rates in wild-type and *fib4* KD apple trees were compared under various levels of incident photosynthetically active radiation (PAR; Fig. 2). At midrange levels of PAR (300 and 600 μE m^{-2} s^{-1}), no significant differences were detected between the net CO_2 fixation rates of wild-type and *fib4* KD trees. Under high levels of PAR (1,000 μE m^{-2} s^{-1}), however, wild-type trees had a significantly higher net CO_2 fixation rate than *fib4* KD trees. Surprisingly, under low levels of PAR (100 μE m^{-2} s^{-1}), the situation was reversed, with *fib4* KD trees having a significantly higher net CO_2 fixation rate than wild-type trees. No significant differences in stomatal conductance were observed between wild-type and

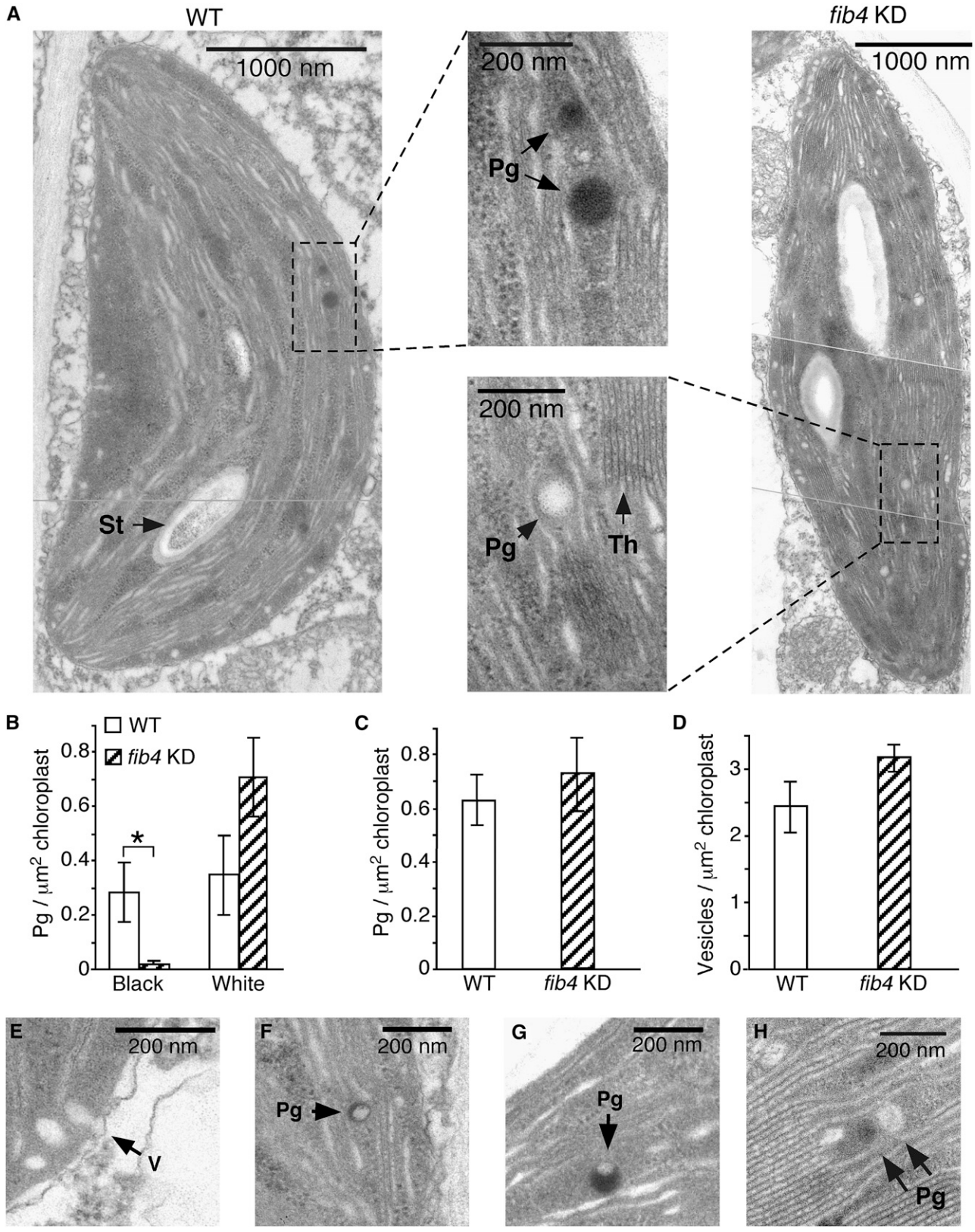

Figure 1. Effects of *FIB4* knockdown on plastoglobule ultrastructure. Trees sampled were 4 to 5 months old and grown under 90 μE m^{-2} s^{-1} light intensity with a 12-h photoperiod. A, Transmission electron micrographs of representative wild-type (WT) and *fib4* KD palisade mesophyll cell chloroplasts stained with OsO_4. Pg, Plastoglobule; St, starch body; Th, thylakoid membrane. For B, C,

fib4 KD trees; however, differences in internal [CO_2] consistent with differences in net CO_2 assimilation rates were observed in *fib4* KD compared with wild-type trees (Supplemental Fig. S3).

Sensitivity to Increases in Light Intensity

The results of the photosynthesis measurements suggested that *fib4* KD apple trees were sensitive to high light. Plants typically accumulate anthocyanins in response to high light stress as photoprotectants (for review, see Steyn et al., 2002). Under a light intensity of 90 μE m^{-2} s^{-1} in plant growth chambers with a 12-h photoperiod, the leaves of *fib4* KD and wild-type apple trees were green and had similar levels of anthocyanins, chlorophyll *a*, chlorophyll *b*, and carotenoids (Fig. 3A; Supplemental Fig. S4). However, following transfer to 600 μE m^{-2} s^{-1} light intensity conditions with a 12-h photoperiod for 6 d, *fib4* KD tree leaves were visibly darker in color than leaves of wild-type trees (Supplemental Fig. S4) and accumulated higher amounts of anthocyanins than wild-type tree leaves, while levels of other pigments were similar (Fig. 3B).

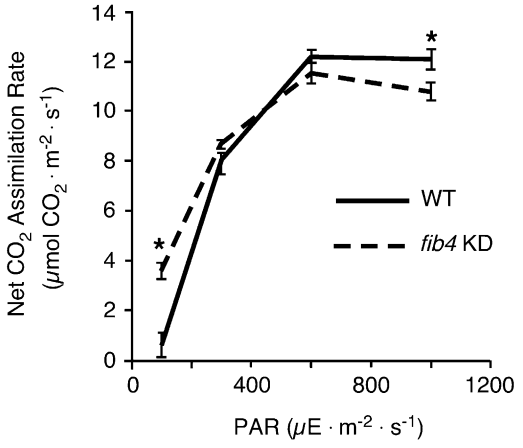

Figure 2. Effects of *FIB4* knockdown on apple tree net photosynthetic CO_2 assimilation rates. Net CO_2 assimilation rates (μmol CO_2 m^{-2} s^{-1}) in wild-type (WT) and *fib4* KD apple tree leaves at various levels of PAR are shown. For each genotype, measurements were taken from three leaves from each of five plants growing in a greenhouse with natural photoperiod and light intensity. Data are means ± SE; * $P < 0.05$ using Student's *t* test.

Methyl Viologen Sensitivity

The sensitivity of *fib4* KD apple trees to high light and increases in light intensity led us to suspect that they might be more sensitive to ROS generation in the chloroplast. Therefore, we tested the sensitivity of *fib4* KD apple trees to methyl viologen (MV). MV generates ROS in chloroplasts by preferential electron acceptance by MV, rather than by ferredoxin, from the reaction center of PSI, producing MV radical, whose oxidation by oxygen produces the superoxide radical $O_2^{\cdot-}$ (Izawa, 1980; Ashton and Crafts, 1981; Taiz and Zeiger, 2006), thereby inducing oxidative damage. When floated on 0.5 μM MV solution for 24 h, leaf discs from *fib4* KD apple trees developed obviously larger areas of brown pigmentation compared with leaf discs from wild-type and VC trees (Fig. 4A). Little or no brown pigmentation was observed on most of the wild-type and VC leaf discs after MV treatment; in contrast, all *fib4* KD leaf discs developed extensive brown coloration. When observed, brown pigmentation in wild-type and VC leaf discs was generally restricted to limited areas near the disc margins. In addition, tissue damage as measured by electrolyte leakage was greater in *fib4* KD leaf discs than wild-type or VC leaf discs at 24 h after the start of MV treatment (Fig. 4B). The greater sensitivity to MV was accompanied by higher levels of superoxide during the course of MV treatment (Fig. 4C), as detected by in situ staining of leaf discs with nitroblue tetrazolium (NBT) using the method described by Garmier et al. (2007). At 3 h after the start of MV treatment, leaf discs from *fib4* KD trees exhibited measurably darker NBT staining than leaf discs from wild-type trees (Fig. 4C). This indicates that *fib4* KD trees had higher levels of superoxide during MV treatment than wild-type trees. Apple showed a high, stable background signal in the superoxide assay; however, the signal increase in *fib4* KD was clear. These results suggest that *fib4* KD trees were more sensitive to oxidative stress.

Susceptibility to Bacterial Pathogens

Oxidative damage also plays a role in disease processes, such as fire blight (Venisse et al., 2001). Therefore, the susceptibility of *fib4* KD apple trees to *E. amylovora* was tested. One of the major symptoms of

Figure 1. (*Continued.*)
and D, 80 chloroplasts from six leaves (two leaves from each of three plants) were observed for each genotype; data are means ± SE; * $P < 0.05$ using Student's *t* test. B, Wild-type chloroplasts had more electron-opaque (black) plastoglobules than *fib4* KD chloroplasts; the numbers of electron-transparent (white) plastoglobules were not significantly different between wild-type and *fib4* KD chloroplasts. Partially and completely black plastoglobules were counted as electron opaque; plastoglobules scored as electron transparent had no electron-opaque sectors. C, Wild-type and *fib4* KD chloroplasts had similar total numbers of plastoglobules (electron opaque + electron transparent). D, Wild-type and *fib4* KD chloroplasts had similar numbers of peripheral vesicles. E, Transmission electron micrograph of peripheral vesicles (V). F, Electron-opaque plastoglobule in a wild-type chloroplast with less than 50% of its area appearing black. G, Electron-opaque plastoglobule in a wild-type chloroplast with more than 50% of its area appearing black. H, Adjacent electron-opaque and electron-transparent plastoglobules in a *fib4* KD chloroplast.

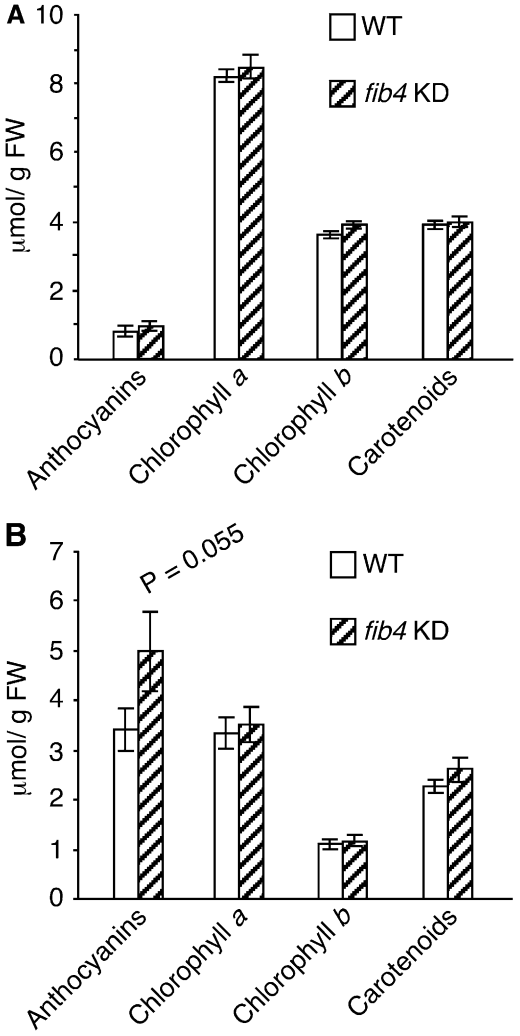

Figure 3. Effects of *FIB4* knockdown on apple tree leaf major pigment levels. A, Major pigment levels in leaves of wild-type (WT) and *fib4* KD plants grown in a growth chamber with 90 μE m^{-2} s^{-1} light intensity and a 12-h photoperiod. Data are from one representative experiment; data are means ± SE; $n = 6$ per genotype/experiment. No statistically significant differences at $P < 0.05$ between wild-type and *fib4* KD plants were observed for any pigment under these conditions using Student's *t* test. B, Major pigment levels in leaves of wild-type and *fib4* KD plants at 6 d after transfer from a growth chamber with 90 μE m^{-2} s^{-1} light intensity and a 12-h photoperiod to a growth room having 600 μE m^{-2} s^{-1} light intensity and a 12-h photoperiod. Data are means ± SE; $n = 6$ per genotype. FW, Fresh weight.

fire blight is a zone of darkened, shriveled, necrotic tissue that develops and spreads systemically from a point of initial infection. Necrotic region length as a percentage of total shoot length is a standard, quantitative measure of fire blight disease severity (Lee et al., 2010). Wild-type, VC, and *fib4* KD apple trees growing in soil in a growth chamber were inoculated with 10^8 colony-forming units (cfu) mL^{-1} *E. amylovora* strain Ea581a at wounded shoot tips. Fire blight disease severity was measured once each week for 3 weeks following inoculation. At each time point, fire blight disease severity was significantly higher in *fib4* KD apple trees than in either wild-type or VC trees (Fig. 5A). In addition, *fib4* KD apple tree shoots supported significantly larger *E. amylovora* population sizes than wild-type shoots (Fig. 5B). These results indicated that *fib4* KD apple trees were more susceptible to *E. amylovora* than control trees.

To determine whether plant immune responses were altered in *fib4* KD apple trees during fire blight infection, we analyzed the expression of *Pathogenesis-Related* (*PR*) genes in *E. amylovora*-infected apple trees. *PR* gene expression is induced in apple trees during *E. amylovora* infection (Venisse et al., 2002). Our results indicated that expression of *PR2* and *PR8* was induced in control and *fib4* KD plants; however, induction was significantly less in *fib4* KD apple trees compared with wild-type trees (Supplemental Fig. S5).

To further study the role of FIB4 in disease resistance, effects of mutations in the orthologous Arabidopsis *FIB4* gene (At3g23400) on bacterial disease resistance were determined. For these experiments, two transgenic Arabidopsis lines homozygous for T-DNA mutations in *FIB4* (*fib4-1* and *fib4-2*) were obtained and characterized (Supplemental Fig. S6). *Pseudomonas syringae* pv *tomato* (*P. s. t.*) causes bacterial speck disease in Arabidopsis (Whalen et al., 1991). Symptoms observed on the leaves of susceptible Arabidopsis plants include tissue necrosis, water-soaked lesion development, and chlorosis. Wild-type ecotype Columbia-0 (Col-0) and *fib4-1* mutant plants were challenged with the virulent *P. s. t.* strain DC3000 by leaf infiltration, and symptom development and bacterial growth were monitored. Bacterial speck disease symptoms were visibly more severe on *fib4-1* than Col-0 at 6 d post inoculation (dpi) with 10^4 cfu mL^{-1} *P. s. t.* (Fig. 5C). Significantly greater electrolyte leakage was recorded for *fib4-1* than Col-0 at 6 dpi with 10^4 cfu mL^{-1} *P. s. t.* (Fig. 5D), indicating more extensive tissue damage in the mutant. In addition, the *P. s. t.* population size in *fib4-1* plant leaves was double that in Col-0 leaves at 4 dpi with 10^4 cfu mL^{-1} bacterium (Fig. 5E). These results show that the Arabidopsis *fib4-1* mutant was more susceptible to bacterial speck disease than wild-type control plants, although the change in bacterial growth was relatively modest compared with some other Arabidopsis mutants that support increased *P. s. t.* growth, such as *sid2* (Block et al., 2005), *eds1*, and *pad4* (Xing and Chen, 2006).

Ozone Sensitivity

The sensitivity of *fib4* KD trees to MV, high light intensity, and bacterial pathogens prompted us to test whether *fib4* KD trees were sensitive to ozone (O_3), another oxidative stress. Exposure to O_3 triggers tissue necrosis and cell death in sensitive plants (Runeckles and Chevonne, 1992). Wild-type and *fib4* KD apple trees were exposed to 350 nL L^{-1} O_3 for 6 h. At 6 h after the start of the O_3 treatment, none of the apple trees had

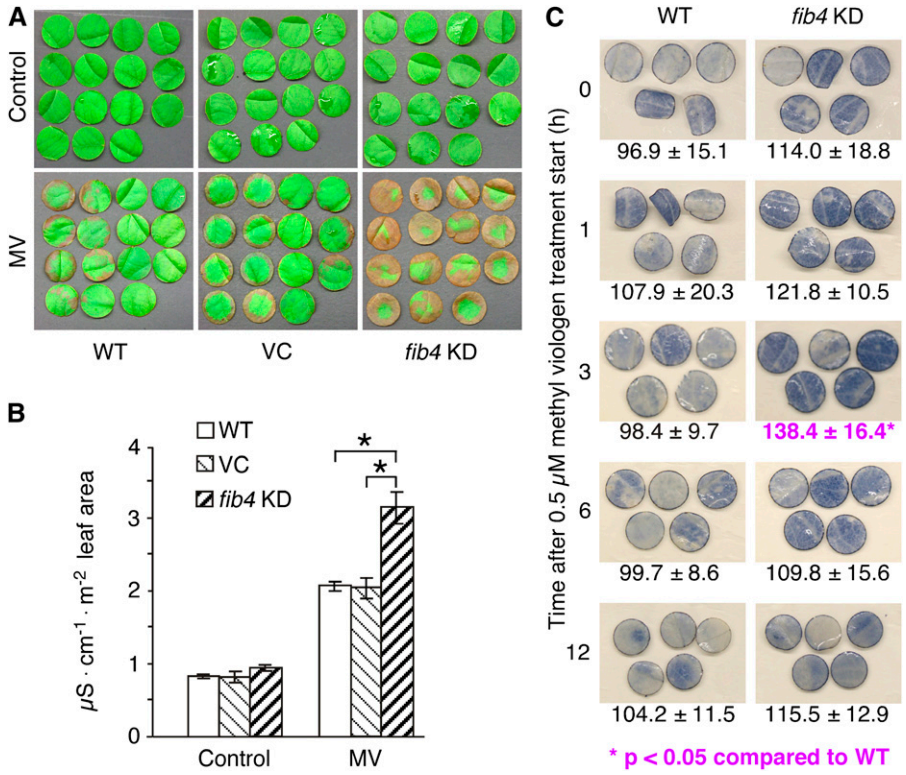

Figure 4. Knockdown of *FIB4* increases apple sensitivity to MV. Leaf discs for each experiment were taken from plants growing in a growth chamber with 90 μE m^{-2} s^{-1} light intensity and a 12-h photoperiod. A, Wild-type (WT), VC, and *fib4* KD apple tree leaf discs after exposure to 0.5 μM MV under continuous 50 μE m^{-2} s^{-1} light for 24 h. Photographs are from one representative experiment; the experiment was repeated three times with similar results; $n = 15$ discs per genotype, two source plants per genotype. B, Electrolyte leakage from wild-type and *fib4* KD leaf discs after exposure to 0.5 μM MV for 24 h under continuous 50 μE m^{-2} s^{-1} light. Electrolyte leakage was measured at 24 h after the beginning of treatment. Data are means ± SE of six replicates; * $P < 0.05$ using Student's *t* test. C, Superoxide radical detection in wild-type and *fib4* KD leaf discs floated on 0.5 μM MV under continuous 50 μE m^{-2} s^{-1} light. Leaf discs were floated on 0.5 μM MV for the indicated times, stained with NBT, and then cleared with ethanol (Garmier et al., 2007). NBT staining intensity was measured using ImageJ software (Abramoff et al., 2004). The experiment was performed using leaf discs from five trees per genotype. Statistical significance was determined using Student's *t* test.

necrotic lesions. However, at 24 h after the start of O_3 treatment, *fib4* KD apple trees developed extensive necrosis, especially in the older leaves, while wild-type and VC trees displayed little or no necrosis (Fig. 6A). Tissue damage as measured by electrolyte leakage was significantly greater in *fib4* KD apple trees than in wild-type and VC apple trees at 24 h after the start of the O_3 treatment (Fig. 6B). These results indicate that *fib4* KD apple trees were much more sensitive to O_3 than control wild-type and VC trees.

Arabidopsis *fib4* mutants were also more sensitive to O_3. Exposure of homozygous Arabidopsis *fib4-1* mutants and Col-0 control plants to 500 nL L^{-1} O_3 for 6 h or 700 nL L^{-1} O_3 for 3 h resulted in higher numbers of leaves with visible lesions per plant in *fib4-1* than in Col-0 at 24 h after the start of O_3 exposure (Fig. 6, C and D). Tissue damage as measured by electrolyte leakage was greater in *fib4-1* and *fib4-2* mutant plants than in Col-0 plants at 6 h after the start of a 3-h, 500 nL L^{-1} O_3 treatment (Fig. 6E; Supplemental Fig. S7). These results indicate that Arabidopsis *fib4-1* and *fib4-2* mutant plants were more sensitive to O_3 than Col-0, which was consistent with the increased O_3 sensitivity of *fib4* KD apple trees.

Plastoglobule Ultrastructure during O_3 Stress

In order to determine whether O_3 stress caused any changes in plastoglobule ultrastructure, wild-type and *fib4* KD apple trees were exposed to 350 nL L^{-1} O_3 for 6 h, leaf tissue was collected and fixed at 2 d after the beginning of O_3 exposure, and plastoglobules in palisade mesophyll cells were examined by TEM. In wild-type plants, the proportion of electron-transparent plastoglobules increased from 4% of total plastoglobules before O_3 treatment to 43% of total plastoglobules after O_3 treatment (Fig. 7, A and B). There were fewer electron-opaque plastoglobules and more total plastoglobules in wild-type plants after O_3 treatment, although the differences were not statistically signifi-

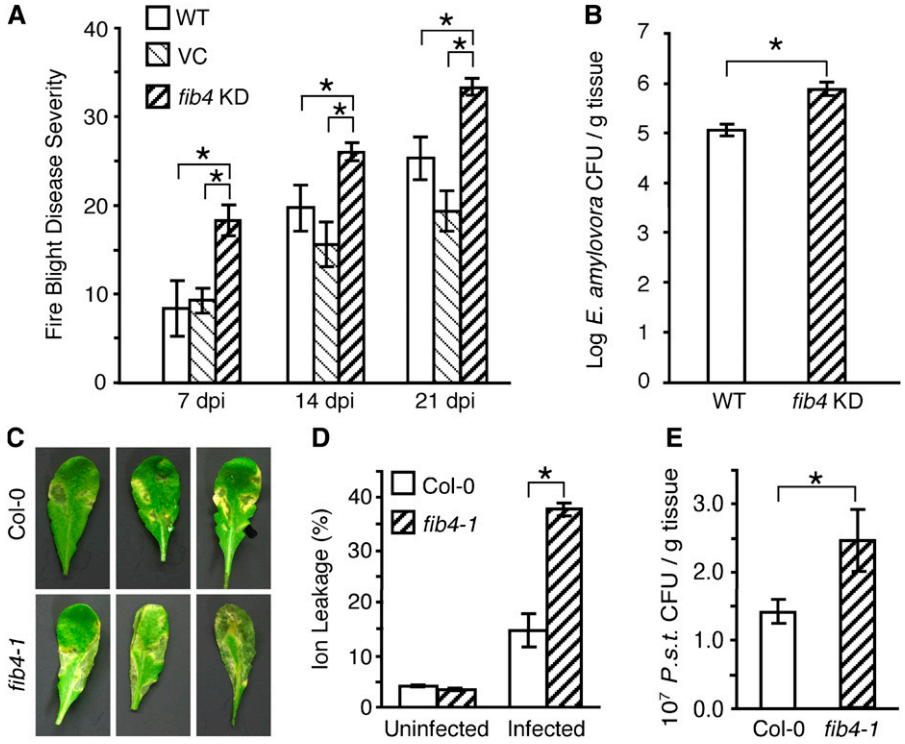

Figure 5. Knockdown or mutation of *FIB4* causes increased susceptibility to bacterial pathogens in apple and Arabidopsis. A and B represent experiments done in apple; C, D, and E represent experiments done in Arabidopsis. All plants were maintained under 90 μE m^{-2} s^{-1} light intensity, with a 12- and 10-h photoperiod for apple and Arabidopsis, respectively, for the duration of the experiments. A, Fire blight disease severity (necrotic zone size) in wild-type (WT), VC, and *fib4* KD trees at 7, 14, and 21 dpi with 10^8 cfu mL^{-1} *E. amylovora*. Data are from one representative experiment; the experiment was repeated four times with similar results. Data are means ± SE; $n = 8$ per genotype/experiment; * $P < 0.05$ using Student's *t* test. B, *E. amylovora* population levels in wild-type and *fib4* KD tree stems at 7 dpi. Data are means ± SE; $n = 4$ or more per genotype; * $P < 0.05$ using Student's *t* test. C, Symptom development on Col-0 and *fib4-1* plant leaves at 6 dpi with 10^4 cfu mL^{-1} *P. s. t.* D, Ion leakage from Col-0 and *fib4-1* leaves at 6 dpi with 10^4 cfu mL^{-1} *P. s. t.* Data are from one representative experiment; the experiment was repeated three times with similar results. Data are means ± SE; $n = 5$ per genotype/experiment; * $P < 0.05$ using Student's *t* test. E, *P. s. t.* population levels in Col-0 and *fib4-1* mutant plant leaves at 4 dpi with 10^4 cfu mL^{-1} bacterium. Data are from one representative experiment; the experiment was repeated three times with similar results. Data are means ± SE; $n = 5$ per genotype/experiment; * $P < 0.05$ using Student's *t* test.

cant (Fig. 7B). In *fib4* KD plants, electron-opaque plastoglobules were virtually absent both before and after O_3 treatment, as expected; however, the total number of plastoglobules in *fib4* KD increased by 56% after O_3 treatment due to an increase in the number of electron-transparent plastoglobules (Fig. 7, A and C).

DISCUSSION

This study indicates that FIB4 is required for plant resistance to several abiotic stresses and for resistance to bacterial pathogens. This finding is consistent with the growing body of evidence linking fibrillins with stress tolerance and disease resistance (Pruvot et al., 1996; Gillet et al., 1998; Rey et al., 2000; Rorat et al., 2001; Cooper et al., 2003; Leitner-Dagan et al., 2006; Yang et al., 2006; Youssef et al., 2010). It is interesting that FIB4 was found to be required for resistance to *P. s. t.*, as FIB4 is one of a handful of proteins that is phosphorylated early on during defense responses against this bacterium, suggesting a role for FIB4 in basal defenses (Jones et al., 2006). The phenotypic similarities between Arabidopsis *fib4* mutants and *fib4* KD apple trees indicate that the function of FIB4 is conserved between these two very different species. This implies that FIB4 may play a basic, conserved role in plant physiology, perhaps as a contributor to plant stress tolerance and/or photosynthetic acclimatization.

This study also indicates that FIB4 is involved in plastoglobule development. While FIB4 does not appear to be required for the production of plastoglobules, the dramatic decrease in plastoglobule osmiophilicity in *fib4* KD apple tree leaf chloroplasts compared with the wild type under benign conditions suggests that FIB4 is required for the development of plastoglobule osmiophilic content. The reactivity of

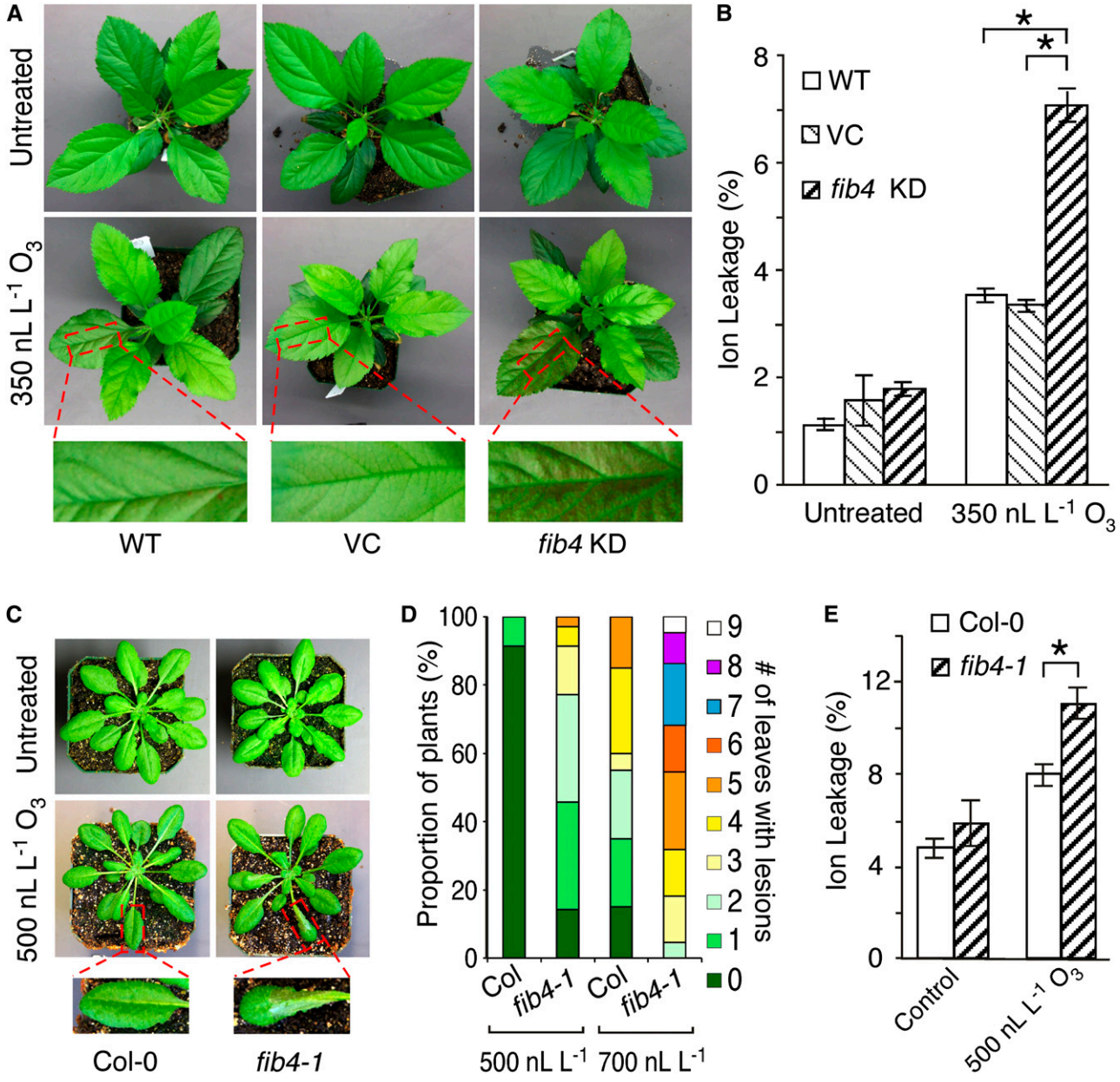

Figure 6. Knockdown and mutation of *FIB4* increases sensitivity to O_3 in apple and Arabidopsis. A and B represent experiments done in apple; C, D, and E represent experiments done in Arabidopsis. A, Effects of exposure to 350 ± 50 nL L^{-1} O_3 for 6 h under 90 μE m^{-2} s^{-1} light intensity on wild-type (WT), VC, and *fib4* KD plants. Plants were returned to their normal light conditions (90 μE m^{-2} s^{-1}, 10-h photoperiod) after O_3 treatment; photographs were taken 24 h after the start of O_3 treatment. B, Ion leakage from wild-type, VC, and *fib4* KD leaves caused by exposure to 350 ± 50 nL L^{-1} O_3 for 6 h under 90 μE m^{-2} s^{-1} light intensity. Plants were returned to their normal light conditions (90 μE m^{-2} s^{-1}, 10-h photoperiod) after O_3 treatment; measurements were taken at 24 h after the start of O_3 treatment. Data are from one representative experiment; the experiment was repeated three times with similar results. Data are means ± SE; $n = 5$ per genotype/experiment; * $P < 0.05$ using Student's *t* test. C, Effects of exposure to 500 ± 50 nL L^{-1} O_3 for 6 h under 90 μE m^{-2} s^{-1} light intensity on Col-0 and *fib4-1* plants. Plants were returned to their normal light conditions (90 μE m^{-2} s^{-1}, 10-h photoperiod) after O_3 treatment; photographs were taken 24 h after the start of O_3 treatment. D, Amount of lesion formation in Col-0 and *fib4-1* plants following exposure to 500 ± 50 nL L^{-1} O_3 for 6 h or 700 ± 50 nL L^{-1} O_3 for 3 h under 90 μE m^{-2} s^{-1} light intensity. Plants were returned to their normal light conditions (90 μE m^{-2} s^{-1}, 10-h photoperiod) after O_3 treatment; measurements were taken at 24 h after the start of O_3 treatment. The percentage of plants with the indicated numbers of lesion-bearing leaves is shown. The number of lesion-bearing leaves per plant ranged from zero to nine. $n = 35$ plants per genotype for plants exposed to 500 ± 50 nL L^{-1} O_3; $n > 20$ plants per genotype for plants exposed to 700 ± 50 nL L^{-1} O_3. E, Ion leakage from Col-0 and *fib4-1* plant leaves after exposure to 500 ± 50 nL L^{-1} O_3 for 3 h under 90 μE m^{-2} s^{-1} light intensity. Plants were returned to their normal light conditions (90 μE m^{-2} s^{-1}, 10-h photoperiod) after O_3 treatment; measurements were taken at 6 h after the start of O_3 treatment. Data are means ± SE; $n = 5$ per genotype; * $P < 0.05$ using Student's *t* test.

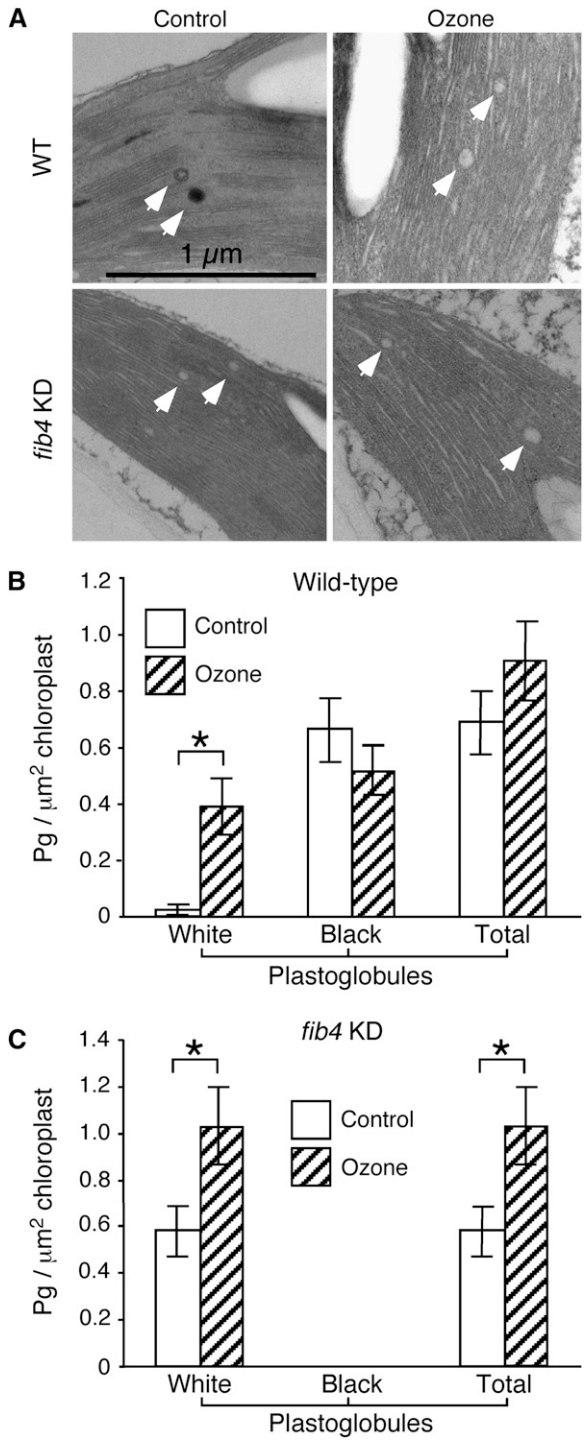

Figure 7. Plastoglobule ultrastructure changes in wild-type and *fib4* KD following O_3 exposure. Wild-type (WT) and *fib4* KD apple trees were exposed to 350 nL L^{-1} O_3 for 6 h under 90 μE m^{-2} s^{-1} light intensity and then returned to their normal growth conditions (90 μE m^{-2} s^{-1}, 10-h photoperiod). Leaf tissues were collected at 2 d after the beginning of the O_3 exposure and examined by TEM. Data shown are from one experiment; the experiment was performed twice with similar results each time. A, Transmission electron micrographs of representative plastoglobules (arrows) in control and O_3-treated wild-type and *fib4* KD leaf palisade mesophyll cell chloroplasts. The scale is the same in all four micrographs. B, Numbers of electron-transparent plastoglobules (white), electron-opaque plastoglobules (black), and total plastoglobules (black + white) per μm^2 in wild-type chloroplasts before and after O_3 exposure. Plastoglobules were counted in 14 chloroplasts from a control plant and 11 chloroplasts from an O_3-exposed plant. Data are means ± SE; * $P < 0.05$ using Student's *t* test. C, Numbers of black and white plastoglobules and total plastoglobules per μm^2 in *fib4* KD chloroplasts before and after O_3 exposure. Plastoglobules were counted in 15 chloroplasts from a control plant and 15 chloroplasts from an O_3-exposed plant. Data are means ± SE; * $P < 0.05$ using Student's *t* test.

osmium oxides with certain biological materials was noted upon the discovery of osmium (Tennant, 1804). Some major plastoglobule constituents such as plastoquinone, carotenoids (Bahr, 1954), and triacylglycerides are expected to contribute to plastoglobule osmiophilicity because their ethylenic double bonds reduce OsO_4, probably producing stable diester adducts (Wigglesworth, 1957) that scatter electrons due to the high mass density and atomic number of osmium (Valentine, 1958). In addition, the reducing power of tocopherols and plastoquinone may contribute to plastoglobule osmiophilicity (Dahlin and Ryberg, 1986). Under mild conditions, FIB4 appears to play a role in the accumulation of osmiophilic material within plastoglobules. However, FIB4 cannot be the sole factor required for plastoglobule osmiophilicity, since osmiophilic plastoglobules were observed in *fib4* KD trees, albeit much fewer than in wild-type apples.

Our finding that plastoglobule osmiophilicity decreased in wild-type apple chloroplasts following acute O_3 stress was consistent with the reduced plastoglobule osmiophilicity observed in earlier studies of chronic O_3 exposure in radish (*Raphanus sativus*; Miyake et al., 1989) and Norway spruce (*Picea abies*; Kivimäenpää et al., 2003). Decreased plastoglobule osmiophilicity could reflect oxidation (Miyake et al., 1989) or egress of plastoglobule osmiophilic content during O_3 stress. This suggests that plastoglobules may be dynamic reservoirs of antioxidants that dispense their contents as needed during O_3 stress; *fib4* KD plastoglobules, which contain very little osmiophilic material to begin with, may be unable to perform this function. This could account for the increased O_3 sensitivity and oxidative stress sensitivity of *fib4* KD trees compared with wild-type trees. In addition, the increase in total number of plastoglobules in *fib4* KD during O_3 stress may represent a physiological compensation for a dearth of osmiophilic plastoglobules.

The increased sensitivity of *fib4* KD plants to MV implies that they are more sensitive to oxidative stress, particularly in the chloroplast. The higher photosynthesis rates of *fib4* KD trees compared with wild-type trees under low-light conditions, which were used for the MV treatments, would likely have exacerbated oxidative stress due to MV treatment in *fib4* KD trees. The higher level of superoxide in MV-treated *fib4* KD apple leaves than in wild-type apple leaves is consistent

with this scenario. Increases or decreases in chloroplast ROS sensitivity are known to affect MV sensitivity. For example, when chloroplastic ROS-scavenging capacity is inhibited by decreasing the expression of thylakoid ascorbate peroxidase (APX), MV sensitivity increases (Tarantino et al., 2005). Furthermore, increasing chloroplastic ROS-scavenging capacity by overexpressing chloroplastic APX (Murgia et al., 2004) or overexpressing cytosolic APX in the chloroplast (Badawi et al., 2004) reduces sensitivity to MV.

Higher anthocyanin accumulation in the leaves of *fib4* KD apple trees than in wild-type apple trees upon shifting to higher light intensity further supports the notion that *fib4* KD plants are more sensitive to oxidative stress in the chloroplast. Anthocyanins protect photosynthetic systems from high light by sunscreen and antioxidant activities (Smillie and Hetherington, 1999; Hughes et al., 2005; Kytridis and Manetas, 2006). Higher anthocyanin accumulation in the leaves of *fib4* KD apple trees upon shifting to higher light intensity might partially compensate for a lack of FIB4-mediated control of oxidative stress in the chloroplast. In addition, increased photoinhibition in *fib4* KD apple leaves compared with the wild type at high light intensity suggests a reduced ability to control photooxidative damage of the photosynthetic system in *fib4* KD trees. The increased O_3 sensitivity of *fib4* KD apple and *fib4* Arabidopsis plants is also consistent with FIB4 contributing to chloroplast oxidative damage control, since chloroplastic ROS production is an early response of plants to O_3 exposure (Joo et al., 2005).

Similarly, the increased susceptibility of Arabidopsis *fib4-1* mutant plants to bacterial speck disease is consistent with increased sensitivity to oxidative damage. *P. s. t.* induces light-dependent ROS accumulation in susceptible host tissues via the phytotoxic virulence factor coronatine (Ishiga et al., 2009). Furthermore, the chloroplast electron transport inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea, which suppresses chloroplast ROS production (Joo et al., 2005), blocks bacterial speck disease development in tomato cotyledons (Ishiga et al., 2009). This suggests that ROS production in chloroplasts is required for bacterial speck disease development (Ishiga et al., 2009). Thus, FIB4 could contribute to bacterial speck disease resistance by mediating oxidative damage control in the chloroplast.

The increased susceptibility of *fib4* KD apple trees to *E. amylovora* is consistent with an increased sensitivity to oxidative damage, although there is currently no clear link between the chloroplast and fire blight. *E. amylovora* induces superoxide production in host plants during infection, resulting in membrane damage and necrosis (Venisse et al., 2001). The production of superoxide appears to play a role in fire blight disease development, possibly by inducing cell death processes in the host. In addition, *PR* gene expression was partially suppressed in *fib4* KD apple trees during *E. amylovora* infection. *PR* gene expression is normally induced in apple trees during *E. amylovora* infection (Venisse et al., 2002), and some of these genes could play a role in reducing apple susceptibility to *E. amylovora* (Maxson-Stein et al., 2002; Malnoy et al., 2007). These results indicate that *fib4* KD apple trees had weakened resistance to *E. amylovora*. In light of these findings, it is interesting that the *E. amylovora*-secreted virulence protein HrpN (Wei et al., 1992) can physically associate with FIB4 (Song et al., 2002). Although this interaction has not been confirmed in vivo, it might make sense if FIB4 were a target of bacterial effector proteins, since reduction of *FIB4* expression triggered disease susceptibility in this study.

The presence of FIB4 in the plastoglobule, the PSII light-harvesting complex, and the thylakoid membrane system, coupled with the presence of a conserved lipocalin signature in FIB4 (Jones et al., 2006), presents a possible mechanism for FIB4 function in plastoglobule development and oxidative stress. Based on its structural similarity to lipocalins (for review, see Flower et al., 2000), FIB4 may be hypothesized to interact with one or more plastoglobule lipid components, helping to transport lipophilic antioxidants in and out of the plastoglobule. In addition, FIB4 could be involved in the delivery of lipophilic antioxidants to particular locations within the photosynthetic membrane system, such as the PSII light-harvesting complex. When the amount of FIB4 is greatly reduced, such as in the *fib4* KD apple trees, then lipophilic antioxidants may not be properly loaded into plastoglobules under benign conditions and may not be dispensed from plastoglobules to locations where they are needed during stress. This hypothesis would explain the increased sensitivity to oxidative stress and alterations in plastoglobule osmiophilicity observed in the *fib4* KD apple trees.

MATERIALS AND METHODS

Plant Material

Arabidopsis (*Arabidopsis thaliana*) plants were grown as described previously (Jambunathan et al., 2001). Arabidopsis T-DNA insertion mutants were obtained from the Arabidopsis Biological Resource Center, a publicly accessible collection of T-DNA insertion lines (http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/abrchome.htm; Alonso et al., 2003). Transgenic RNAi and control pGH00.0131-EGFP expressing apple (*Malus* × *domestica*) plants were generated as described previously (Maximova et al., 1998). For details of vector construction and transformation to generate *fib4* KD apple plants, see Supplemental Figure S1 and its legend. In vitro rooted apple plants were transferred to potting mix (Redi-Earth) and acclimated in a growth chamber under the same environmental conditions as described for Arabidopsis (Jambunathan et al., 2001).

RNA Extraction, DNA Extraction, and PCR

Total RNA from apple leaves was isolated using an established protocol (Jensen et al., 2010). Total RNA from Arabidopsis leaves was isolated as described previously (Jambunathan et al., 2001). DNA was eliminated from RNA samples using the DNA-Free kit (Ambion). Conventional reverse transcription-PCR was performed as described previously (Liu et al., 2005; for primers, see Supplemental Table S1). Quantitative real-time PCR analyses

were performed at the Pennsylvania State University Genomics Core Facility as described previously (Jensen et al., 2010; for primers and probes, see Supplemental Table S2). For *PR* gene expression analyses, apple inoculations with *Erwinia amylovora* and tissue collection were done as described by Norelli et al. (2009) using gene-specific primers for each *PR* gene (for primers, see Supplemental Table S2). Genomic DNA was isolated from young leaves using standard methods (Kasajima et al., 2004). PCR was performed using the Advantage 2 PCR enzyme system kit according to the manufacturer's instructions (Clontech). The LBb1.3 primer sequence was obtained from http://signal.salk.edu/tdnaprimers.html.

TEM

For the TEM experiments presented in Figure 1, leaf discs were collected from fully expanded leaves of 4- to 5-month-old apple plants acclimated in the growth chamber with 90 μE m^{-2} s^{-1} light intensity and a 12-h photoperiod. Tissues were fixed with glutaraldehyde in HEPES buffer, postfixed in OsO_4 in the same buffer, and embedded in Spurr's resin, according to the method of Owen and Makaroff (1995), essentially as described by Li et al. (2004). Ultrathin sections (80 nm thick) were cut using a Reichart-Jung Ultracut E Microtome and stained with uranyl acetate and lead citrate. Observations of chloroplasts were made with a JEOL 1200 EX II transmission electron microscope fitted with a high-resolution Tietz F224 digital camera. Chloroplast areas were measured using ImageJ software (http://rsbweb.nih.gov/ij/). Plastoglobules were counted independently by two people, and one count was done as a blind experiment. For the O_3 TEM experiments presented in Figure 7, 1- to 2-month-old apple trees growing in a growth room with 90 μE m^{-2} s^{-1} light intensity and a 10-h photoperiod were exposed to 350 nL L^{-1} O_3 for 6 h. Leaf tissues were collected at 2 d after the beginning of the O_3 exposure and examined by TEM, and plastoglobules were counted.

Photosynthesis Measurements

Photosynthesis measurements were carried out on potted *fib4* KD and wild-type trees maintained at 22°C ± 3°C in a greenhouse. The second, third, and fourth fully expanded leaves on each of five replicate trees were used for measurements, and the readings were repeated three times for each of the experiments. Measurements were taken on the center of each leaf. For both light intensity and intercellular [CO_2] experiments, measurements were taken on consecutive days between 10:00 AM and 2:00 PM on each day. Gas-exchange measurements were taken with a CIRAS-I infrared gas analyzer equipped with a 2.5-cm^2 Parkinson (PLC 6U) leaf cuvette with light-emitting diode lights (PP Systems). Light at the indicated intensity was applied to the leaves, and net CO_2 assimilation, stomatal conductance, and intercellular [CO_2] were measured.

Pigment Analysis

Anthocyanins, carotenoids, chlorophyll *a*, and chlorophyll *b* were extracted from leaves and measured as described by Sims and Gamon (2002).

Light, O_3, and Herbicide Treatments

O_3 treatments were performed in a tissue culture chamber (model CU-32L; Percival Scientific) attached to an O_3 generator (model 2000; Jelight Company) and an O_3 monitor (model 450; Advanced Pollution Instrumentation). High-light treatment consisted of exposure to 600 μE m^{-2} s^{-1} for 6 d at 21°C with a 12-h photoperiod and 35% to 45% relative humidity. For herbicide treatments, apple tree leaf discs were cut with a 1-cm-diameter cork borer and floated on 20 mL of water or 0.5 μM MV (Sigma) in a petri dish for 24 h. Fifteen leaf discs representing two individual plants (seven or eight discs per plant) were used per sample, and six biological replicates were done for each plant genotype for each treatment.

Disease Assays

Pseudomonas syringae infection and population analysis in Arabidopsis was done using a published protocol (Jambunathan et al., 2001). Apple infection with *E. amylovora* and disease severity measurement was done as described previously (Jensen et al., 2003). Bacterial populations in apple tissue were determined by tissue extraction and serial dilution plating.

Electrolyte Leakage Assays

For the MV experiments, the conductivity of the MV treatment and control solutions (in μS cm^{-1}) was measured at 24 h after the beginning of the experiment using a Corning 316 conductivity meter and divided by the leaf area used in the sample (in m^2). Relative ion leakage (Joo et al., 2005) was used for the bacterial speck disease and O_3 experiments. Apple or Arabidopsis leaves were collected, rinsed with deionized water, and immersed in 25 mL of deionized water for 4 h with shaking at 100 rpm, and the conductivity (μS cm^{-1}) of the resulting solution was measured. The samples were then autoclaved, and conductivity was measured again. Relative ion leakage was calculated by dividing the conductivity of the electrolyte leakage solution before autoclaving by the conductivity of the electrolyte solution after autoclaving and multiplying by 100.

Detection of Superoxide

In situ O_2^- was detected using a NBT staining method essentially as described by Garmier et al. (2007). In brief, MV-treated leaf discs were vacuum infiltrated with a solution of 0.5 mg mL^{-1} NBT (Sigma) in 10 mM potassium phosphate buffer (pH 7.8) and kept in dark for 1 h, and then chlorophyll was cleared by immersion in ethanol at 70°C. Leaf discs were photographed using a FinePix S9000 digital camera (FujiFilm), and NBT staining intensity was measured using ImageJ software (Abramoff et al., 2004). Each sample consisted of five leaf discs, each disc taken from a different tree.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Construction of the apple *FIB4* RNAi binary vector used for *Agrobacterium tumefaciens*-mediated genetic transformation of apple.

Supplemental Figure S2. Confirmation of reduced expression of the *FIB4* gene in *fib4* KD apple trees, and phenotype of wild-type and transgenic trees.

Supplemental Figure S3. Internal [CO_2] and stomatal conductance of *fib4* KD and wild-type apple trees.

Supplemental Figure S4. Effects of transfer from 90 μE m^{-2} s^{-1} PAR to 600 μE m^{-2} s^{-1} PAR for 6 d on wild-type and *fib4* KD plants.

Supplemental Figure S5. Expression of *PR* genes was induced in wild-type (WT) and *fib4* KD apple tree during *E. amylovora* infection.

Supplemental Figure S6. Confirmation of T-DNA insertion in the Arabidopsis *FIB4* gene.

Supplemental Figure S7. The *fib4-1* and *fib4-2* mutants are more sensitive to O_3 than wild-type Col-0 plants.

Supplemental Table S1. PCR primer sequences.

Supplemental Table S2. Real-time PCR primer and probe sequences.

ACKNOWLEDGMENTS

We thank Dr. Mark Guiltinan (The Pennsylvania State University) for providing tissue culture laboratory and plant growth facilities for the generation and maintenance of the transgenic apple plants, Ann Young (The Pennsylvania State University) for help with the construction of pGY06.0228, and Sharon Pishak (The Pennsylvania State University) for her support in generating the transgenic apple plants. We thank Dr. Herb S. Aldwinckle (Cornell University) for providing the cv Royal Gala tissue culture lines used for transformation. We thank Ruth Haldeman, Missy Hazen, and Greg Ning at the Pennsylvania State University Electron Microscopy Facility at University Park for providing training and support needed to use the transmission electron microscope. We thank Sherry Coven (The Pennsylvania State University) for assistance with plastoglobule counts.

Received August 6, 2010; accepted August 31, 2010; published September 1, 2010.

LITERATURE CITED

Abramoff MD, Magelhaes PJ, Ram SJ (2004) Image processing with ImageJ. Biophotonics Int **11:** 36–42

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science **301:** 653–657

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol **55:** 373–399

Asada K (2006) Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol **141:** 391–396

Ashton FM, Crafts AS (1981) Mode of Action of Herbicides, Ed 2. Wiley, New York

Austin JR II, Frost E, Vidi P-A, Kessler F, Staehelin LA (2006) Plastoglobules are lipoprotein subcompartments of the chloroplast that are permanently coupled to thylakoid membranes and contain biosynthetic enzymes. Plant Cell **18:** 1693–1703

Badawi GH, Kawano N, Yamauchi Y, Shimada E, Sasaki R, Kubo A, Tanaka K (2004) Over-expression of ascorbate peroxidase in tobacco chloroplasts enhances the tolerance to salt stress and water deficit. Physiol Plant **121:** 231–238

Bahr GF (1954) Osmium tetroxide and ruthenium tetroxide and their reactions with biologically important substances: electron stains III. Exp Cell Res **7:** 457–479

Block A, Schmelz E, Jones JB, Klee HJ (2005) Coronatine and salicylic acid: the battle between Arabidopsis and *Pseudomonas* for phytohormone control. Mol Plant Pathol **6:** 79–83

Bréhélin C, Kessler F, van Wijk KJ (2007) Plastoglobules: versatile lipoprotein particles in plastids. Trends Plant Sci **12:** 260–266

Cooper B, Clarke JD, Budworth P, Kreps J, Hutchison D, Park S, Guimil S, Dunn M, Luginbühl P, Ellero C, et al (2003) A network of rice genes associated with stress response and seed development. Proc Natl Acad Sci USA **100:** 4945–4950

Dahlin C, Ryberg H (1986) Accumulation of phytoene in plastoglobuli of SAN-9789 (norflurazon)-treated dark-grown wheat. Physiol Plant **68:** 39–45

Deruère J, Römer S, d'Harlingue A, Backhaus RA, Kuntz M, Camara B (1994) Fibril assembly and carotenoid overaccumulation in chromoplasts: a model for supramolecular lipoprotein structures. Plant Cell **6:** 119–133

Emter O, Falk H, Sitte P (1990) Specific carotenoids and proteins as prerequisites for chromoplast tubule formation. Protoplasma **157:** 128–135

Flower DR, North ACT, Sansom CE (2000) The lipocalin protein family: structural and sequence overview. Biochim Biophys Acta **1482:** 9–24

Friso G, Giacomelli L, Ytterberg AJ, Peltier J-B, Rudella A, Sun Q, van Wijk KJ (2004) In-depth analysis of the thylakoid membrane proteome of *Arabidopsis thaliana* chloroplasts: new proteins, new functions, and a plastid proteome database. Plant Cell **16:** 478–499

Galetskiy D, Susnea I, Reiser V, Adamska I, Przybylski M (2008) Structure and dynamics of photosystem II light-harvesting complex revealed by high-resolution FTICR mass spectrometric proteome analysis. J Am Soc Mass Spectrom **19:** 1004–1013

Garmier M, Priault P, Vidal G, Driscoll S, Djebbar R, Boccara M, Mathieu C, Foyer CH, De Paepe R (2007) Light and oxygen are not required for harpin-induced cell death. J Biol Chem **282:** 37556–37566

Gillet B, Beyly A, Peltier G, Rey P (1998) Molecular characterization of *CDSP 34*, a chloroplastic protein induced by water deficit in *Solanum tuberosum* L. plants, and regulation of *CDSP 34* expression by ABA and high illumination. Plant J **16:** 257–262

Hughes NM, Neufeld HS, Burkey KO (2005) Functional role of anthocyanins in high-light winter leaves of the evergreen herb *Galax urceolata*. New Phytol **168:** 575–587

Ishiga Y, Uppalapati SR, Ishiga T, Elavarthi S, Martin B, Bender CL (2009) The phytotoxin coronatine induces light-dependent reactive oxygen species in tomato seedlings. New Phytol **181:** 147–160

Izawa S (1980) Acceptors and donors for chloroplast electron transport. Methods Enzymol **69:** 413–434

Jambunathan N, Siani JM, McNellis TW (2001) A humidity-sensitive *Arabidopsis* copine mutant exhibits precocious cell death and increased disease resistance. Plant Cell **13:** 2225–2240

Jensen PJ, Makalowska I, Altman N, Fazio G, Praul C, Maximova SN, Crassweller RM, Travis JW, McNellis TW (2010) Rootstock-regulated gene expression patterns in apple tree scions. Tree Genet Genomes **6:** 57–72

Jensen PJ, Rytter J, Detwiler EA, Travis JW, McNellis TW (2003) Rootstock effects on gene expression patterns in apple tree scions. Plant Mol Biol **53:** 493–511

Jones AME, Bennett MH, Mansfield JW, Grant M (2006) Analysis of the defence phosphoproteome of *Arabidopsis thaliana* using differential mass tagging. Proteomics **6:** 4155–4165

Joo JH, Wang S, Chen JG, Jones AM, Fedoroff NV (2005) Different signaling and cell death roles of heterotrimeric G protein α and β subunits in the *Arabidopsis* oxidative stress response to ozone. Plant Cell **17:** 957–970

Kasajima I, Ide Y, Ohkama-Ohtsu N, Hayashi H, Yoneyama T, Fujiwara T (2004) A protocol for rapid DNA extraction from *Arabidopsis thaliana* for PCR analysis. Plant Mol Biol Rep **22:** 49–52

Kivimäenpää M, Sutinen S, Karlsson PE, Selldén G (2003) Cell structural changes in the needles of Norway spruce exposed to long-term ozone and drought. Ann Bot (Lond) **92:** 779–793

Knoth R, Hansmann P, Sitte P (1986) Chromoplasts of *Palisota barteri*, and the molecular structure of chromoplast tubules. Planta **168:** 167–174

Kytridis VP, Manetas Y (2006) Mesophyll versus epidermal anthocyanins as potential *in vivo* antioxidants: evidence linking the putative antioxidant role to the proximity of oxy-radical source. J Exp Bot **57:** 2203–2210

Laizet Y, Pontier D, Mache R, Kuntz M (2004) Subfamily organization and phylogenetic origin of genes encoding plastid lipid-associated proteins of the fibrillin type. J Genome Sci Technol **3:** 19–28

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol **48:** 251–275

Langenkämper G, Manac'h N, Broin M, Cuiné S, Becuwe N, Kuntz M, Rey P (2001) Accumulation of plastid lipid-associated proteins (fibrillin/CDSP34) upon oxidative stress, ageing and biotic stress in Solanaceae and in response to drought in other species. J Exp Bot **52:** 1545–1554

Lee SA, Ngugi HK, Halbrendt NO, O'Keefe G, Lehman B, Travis JW, Sinn JP, McNellis TW (2010) Virulence characteristics accounting for fire blight disease severity in apple trees and seedlings. Phytopathology **100:** 539–550

Leitner-Dagan Y, Ovadis M, Shklarman E, Elad Y, Rav David D, Vainstein A (2006) Expression and functional analyses of the plastid lipid-associated protein CHRC suggest its role in chromoplastogenesis and stress. Plant Physiol **142:** 233–244

Li W, Chen C, Markmann-Mulisch U, Timofejeva L, Schmelzer E, Ma H, Reiss B (2004) The *Arabidopsis* AtRAD51 gene is dispensable for vegetative development but required for meiosis. Proc Natl Acad Sci USA **101:** 10596–10601

Liu J, Jambunathan N, McNellis TW (2005) Transgenic expression of the von Willebrand A domain of the BONZAI 1/COPINE 1 protein triggers a lesion-mimic phenotype in *Arabidopsis*. Planta **221:** 85–94

Liu Y, Ren D, Pike S, Pallardy S, Gassmann W, Zhang S (2007) Chloroplast-generated reactive oxygen species are involved in hypersensitive response-like cell death mediated by a mitogen-activated protein kinase cascade. Plant J **51:** 941–954

Malnoy M, Jin Q, Borejsza-Wysocka EE, He SY, Aldwinckle HS (2007) Overexpression of the apple *MpNPR1* gene confers increased disease resistance in *Malus* × *domestica*. Mol Plant Microbe Interact **20:** 1568–1580

Maximova SN, Dandekar AM, Guiltinan MJ (1998) Investigation of *Agrobacterium*-mediated transformation of apple using green fluorescent protein: high transient expression and low stable transformation suggest that factors other than T-DNA transfer are rate-limiting. Plant Mol Biol **37:** 549–559

Maxson-Stein K, He SY, Hammerschmidt R, Jones AL (2002) Effect of treating apple trees with acibenzolar-*S*-methyl on fire blight and expression of pathogenesis-related protein genes. Plant Dis **86:** 785–790

Mehdy MC (1994) Active oxygen species in plant defense against pathogens. Plant Physiol **105:** 467–472

Miyake H, Matsumura H, Fujinuma Y, Totsuka T (1989) Effects of low concentrations of ozone on the fine structure of radish leaves. New Phytol **111:** 187–195

Murgia I, Tarantino D, Vannini C, Bracale M, Carravieri S, Soave C (2004) *Arabidopsis thaliana* plants overexpressing thylakoidal ascorbate peroxidase show increased resistance to paraquat-induced photooxidative stress and to nitric oxide-induced cell death. Plant J **38:** 940–953

Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active

oxygen under control. Annu Rev Plant Physiol Plant Mol Biol **49:** 249–279

Norelli JL, Farrell RE Jr, Bassett CL, Baldo AM, Lalli DA, Aldwinckle HS, Wisniewski ME (2009) Rapid transcriptional response of apple to fire blight disease revealed by cDNA suppression subtractive hybridization analysis. Tree Genet Genomes **5:** 27–40

Owen HA, Makaroff CA (1995) Ultrastructure of microsporogenesis and microgametogenesis in *Arabidopsis thaliana* (L.) Heynh. ecotype Wassilewskija (Brassicaceae). Protoplasma **185:** 7–21

Padham AK, Hopkins MT, Wang T-W, McNamara LM, Lo M, Richardson LGL, Smith MD, Taylor CA, Thompson JE (2007) Characterization of a plastid triacylglycerol lipase from Arabidopsis. Plant Physiol **143:** 1372–1384

Peltier J-B, Ytterberg AJ, Sun Q, van Wijk KJ (2004) New functions of the thylakoid membrane proteome of *Arabidopsis thaliana* revealed by a simple, fast, and versatile fractionation strategy. J Biol Chem **279:** 49367–49383

Pozueta-Romero J, Rafia F, Houlné G, Cheniclet C, Carde JP, Schantz ML, Schantz R (1997) A ubiquitous plant housekeeping gene, *PAP*, encodes a major protein component of bell pepper chromoplasts. Plant Physiol **115:** 1185–1194

Pruvot G, Cuiné S, Peltier G, Rey P (1996) Characterization of a novel drought-induced 34-kDa protein located in the thylakoids of *Solanum tuberosum* L. plants. Planta **198:** 471–479

Rey P, Gillet B, Römer S, Eymery F, Massimino J, Peltier G, Kuntz M (2000) Over-expression of a pepper plastid lipid-associated protein in tobacco leads to changes in plastid ultrastructure and plant development upon stress. Plant J **21:** 483–494

Rorat T, Havaux M, Irzykowski W, Cuiné S, Becuwe N, Rey P (2001) *PSII-S* gene expression, photosynthetic activity and abundance of plastid thioredoxin-related and lipid-associated proteins during chilling stress in *Solanum* species differing in freezing resistance. Physiol Plant **113:** 72–78

Runeckles VC, Chevonne BI (1992) Crop responses to ozone. *In* AS Lefohn, ed, Surface Level Ozone Exposures and Their Effects on Vegetation. Lewis Publishers, Chelsea, MI, pp 189–270

Sanabria NM, Dubery IA (2006) Differential display profiling of the *Nicotiana* response to LPS reveals elements of plant basal resistance. Biochem Biophys Res Commun **344:** 1001–1007

Shetty NP, Jørgensen HJL, Jensen JD, Collinge DB, Shetty HS (2008) Roles of reactive oxygen species in interactions between plants and pathogens. Eur J Plant Pathol **121:** 267–280

Sims DA, Gamon JA (2002) Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. Remote Sens Environ **81:** 337–354

Smillie RM, Hetherington SE (1999) Photoabatement by anthocyanin shields photosynthetic systems from light stress. Photosynthetica **36:** 451–463

Song X, Fan H, Wei Z-M, inventors. January 17, 2002. Receptors for hypersensitive response elicitors and uses thereof. U.S. Patent Application No. 20020007501

Steinmüller D, Tevini M (1985) Composition and function of plastoglobuli. Planta **163:** 201–207

Steyn WJ, Wand SJE, Holcroft DM, Jacobs G (2002) Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. New Phytol **155:** 349–361

Taiz L, Zeiger E (2006) Mode of action of some herbicides. Plant Physiology. http://4e.plantphys.net/index2.php (August 5, 2010)

Tarantino D, Vannini C, Bracale M, Campa M, Soave C, Murgia I (2005) Antisense reduction of thylakoidal ascorbate peroxidase in *Arabidopsis* enhances paraquat-induced photooxidative stress and nitric oxide-induced cell death. Planta **221:** 757–765

Tennant S (1804) On two metals, found in the black powder remaining after the solution of platina. Philos Trans R Soc Lond **94:** 411–418

Tevini M, Steinmüller D (1985) Composition and function of plastoglobuli. Planta **163:** 91–96

Valentine RC (1958) Contrast in the electron microscope image. Nature **181:** 832–833

Venisse JS, Gullner G, Brisset MN (2001) Evidence for the involvement of an oxidative stress in the initiation of infection of pear by *Erwinia amylovora*. Plant Physiol **125:** 2164–2172

Venisse JS, Malnoy M, Faize M, Paulin JP, Brisset MN (2002) Modulation of defense responses of *Malus* spp. during compatible and incompatible interactions with *Erwinia amylovora*. Mol Plant Microbe Interact **15:** 1204–1212

Vidi PA, Kanwischer M, Baginsky S, Austin JR, Csucs G, Dörmann P, Kessler F, Bréhélin C (2006) Tocopherol cyclase (VTE1) localization and vitamin E accumulation in chloroplast plastoglobule lipoprotein particles. J Biol Chem **281:** 11225–11234

Wei ZM, Laby RJ, Zumoff CH, Bauer DW, He SY, Collmer A, Beer SV (1992) Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science **257:** 85–88

Whalen MC, Innes RW, Bent AF, Staskawicz BJ (1991) Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. Plant Cell **3:** 49–59

Wigglesworth VB (1957) The use of osmium in the fixation and staining of tissues. Proc R Soc Lond B Biol Sci **147:** 185–199

Winkenbach F, Falk H, Liedvogel B, Sitte P (1976) Chromoplasts of *Tropaeolum majus* L.: isolation and characterization of lipoprotein elements. Planta **128:** 23–28

Xing D, Chen Z (2006) Effects of mutations and constitutive overexpression of *EDS1* and *PAD4* on plant resistance to different types of microbial pathogens. Plant Sci **171:** 251–262

Yang Y, Sulpice R, Himmelbach A, Meinhard M, Christmann A, Grill E (2006) Fibrillin expression is regulated by abscisic acid response regulators and is involved in abscisic acid-mediated photoprotection. Proc Natl Acad Sci USA **103:** 6061–6066

Youssef A, Laizet Y, Block MA, Maréchal E, Alcaraz JP, Larson TR, Pontier D, Gaffé J, Kuntz M (2010) Plant lipid-associated fibrillin proteins condition jasmonate production under photosynthetic stress. Plant J **61:** 436–445

Ytterberg AJ, Peltier JB, van Wijk KJ (2006) Protein profiling of plastoglobules in chloroplasts and chromoplasts: a surprising site for differential accumulation of metabolic enzymes. Plant Physiol **140:** 984–997