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Ectopic Overexpression of SIPK in Poplar Renders Plants Hypersensitive to Ozone

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ABSTRACT

Trees respond to a myriad of environmental stresses by activating an array of signalling molecules and genes whose functions enable the plant tissues to retain integrity and to counter the stressors. While a number of stress-induced genes have been identified, there is much less known about the process by which detection of a stress is linked to changes in transcriptional regulation and the ensuing phenotypic outcome. Here, we show that heterologous over-expression of salicylate-induced protein kinase (SIPK), a tobacco mitogen-activated protein kinase (MAPK) involved in transcriptional regulation and oxidant signalling, leads to increased sensitivity of transgenic poplar seedlings to ozone treatment. Ozone treatment of the transgenic poplar also resulted in rapid activation of the ectopically expressed SIPK, as well as the endogenous MAPK[46], with concomitant elevation of leaf-localized H_2O_2 levels. The results indicate a conserved function of MAPKs in regulating oxidant signalling in various plant species.

Keywords: *Agrobacterium tumefaciens*, hydrogen peroxide, mitogen-activated protein kinase, reactive oxygen species, signalling, transgenic trees

Abbreviations: **AT**, *Agrobacterium* (*tumefaciens*)-mediated transformation; **DAB**, 3, 3'-diaminobenzidine; **MAPK**, mitogen-activated protein kinase; **MPK6**, mitogen-activated protein kinase 6; **PCD**, programmed cell death; **ROS**, reactive oxygen species; **SIPK**, salicylic acid-induced protein kinase; **VOC**, volatile organic compounds; **WIPK**, wound-induced protein kinase

INTRODUCTION

Tropospheric ozone is one of the most pervasive and deleterious air pollutants in many areas in the industrialized world (Krupa *et al*. 1988). Over the past 20 years in the United States and Europe, ambient levels of ozone have increased by 1-2% per year (Stockwell *et al*. 1997) with little indication of subsiding. Background tropospheric ozone levels in unpolluted regions can be between 20 to 60 nL L^{-1}, whereas ozone concentrations in urban areas, due to industrial and vehicle emissions of nitrogen oxides NO_x (= NO + NO_2), volatile organic compounds (VOC), and carbon monoxide (CO) can greatly exceed these background levels (Seinfeld 1989; Aneja *et al*. 2000). Forested areas located downwind from these industrialized areas are also at risk to this elevated ozone through horizontal transport, a process which occurs within the troposphere (Beaver and Palazoglu 2009). This scenario results in higher levels of ozone occurring downwind from the original source (Seinfeld 1989; Sillman 1999). Ozone is believed to cause more damage to forest trees in Europe and North America than any other gaseous pollutant (Koch *et al*. 1998; Langebartels *et al*. 1998).

The detrimental effects of this pollutant on trees, as well as other plant life, include, but are not limited to, diminished photosynthesis, reduced biosynthesis of Rubisco, growth retardation, accelerated foliar senescence, programmed cell death (PCD), and altered patterns of carbon allocation (Reich and Amundson 1985; Coleman *et al*. 1995a; Coleman *et al*. 1995b; Brendley and Pell 1998; Riehl *et al*. 2000; Guidi *et al*. 2001; Van Hove *et al*. 2001). These perturbations in the metabolic robustness of trees can lead to an increased susceptibility to biotic and abiotic stressors contributing to forest decline (McLaughlin 1985; Schmieden and Wild 1995).

Ozone is taken up into the leaf interior via the stomata during normal gas exchange (Kerstiens and Lendzian 1989), where it comes in contact with the compounds present in the cell wall, apoplastic fluid and plasmalemma (Sharma and Davis 1997; reviewed in Plöchl *et al*. 2000; Renaut *et al*. 2009). Once ozone comes in contact with these cellular components, it is immediately converted to oxygen-centered, reactive oxygen species (ROS) such as superoxide anion ($^{\bullet}O_2^-$); hydroperoxyl radical ($HO_2^{\bullet}$), the conjugate acid to the superoxide anion; hydroxyl radical ($HO^{\bullet}$); singlet oxygen ($^1O_2$*), and hydrogen peroxide (H_2O_2) (Kanofsky and Sima 1995; Runeckles and Vaartnou 1997; Pellien *et al*. 1999; reviewed in Renaut *et al*. 2009). In birch leaves, it has been shown that the ozone-induced H_2O_2 initially accumulates around the cell's plasmalemma (Pellinen *et al*. 1999) where it has the potential to move across the plasmalemma (Finkel 1998; Branco *et al*. 2004) and oxidatively modify lipids, receptors and other proteins. Since these ROS can be toxic to living cells, plants have, in addition to a basal level of antioxidant metabolites, evolved a complex, enzyme-based oxidant sensing and response system. This helps to counteract the deleterious effects of oxidants derived from various sources, including ozone, photooxidation, and ultra-violet radiation (UVR).

Numerous lines of evidence gathered from genetic and biochemical studies of plant stress signalling indicate that phosphorylation and dephosphorylation of proteins are important in the regulation of gene expression and physiological status in response to a myriad of biotic and abiotic stresses (Ichimura *et al*. 2000). MAPK cascades, which are ubiquitous among eukaryotic organisms (Herskowitz 1995), are highly regulated networks of phosphoproteins arranged in multiple, inter-connecting, hierarchical phosphorelays,

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and are central to an organism's ability to respond to these stresses. Ozone challenge has been shown to rapidly activate MAPK signalling in diverse plant species including tobacco (Samuel *et al*. 2000; Samuel and Ellis 2002) (WIPK and SIPK), Arabidopsis (Miles *et al*. 2005, 2009a, 2009b) (MPK3 and MPK6), poplar (Hamel *et al*. 2005), along with white spruce (*Picea gluaca*), corn (*Zea mays* L.), strawberry (*Fragaria* × *ananassa*), and moss (*Physcomitrella patens*) (Miles and Ellis, unpublished data). Activation of these MAPKs has been shown to be dependent on ROS accumulation, receptor activation, calcium influx and activation of one or more upstream MAPKK(s).

We have previously identified that salicylate-induced protein kinase (SIPK), a MAPK is a central regulator of ozone-induced cell death (Samuel *et al*. 2000). In order to examine its conserved role in a tree species, we created a number of transgenic poplar lines that stably overexpressed SIPK to various levels and tested them for: 1) activation of ectopic SIPK by ozone, and 2) altered sensitivity to ozone. Ozone-induced activation of ectopic SIPK was detected, along with an increased sensitivity of these transgenic plants to ozone. The enhanced sensitivity was also accompanied by elevated leaf-localized H_2O_2 levels.

MATERIALS AND METHODS

Plant material and treatment

Hybrid poplar plants, *Populus tremula* X *Populus alba*, 'INRA 717 1B4' (Leple *et al*. 1992), were maintained *in vitro* in Magenta boxes containing propagation medium [MS salts, MES buffer, *myo*-inositol, L-glutamine, vitamins, sucrose, and phytagel at pH 5.8]. These plants were incubated at 25°C under a 16 hr photoperiod of cool-white fluorescent light (25–32 µmole/sec/m^2). Plants were subcultured every fourth week by aseptically transferring shoot apices to fresh medium.

Ozone was generated in a flow through chamber at 3L/minute, with a Delzone ZO-300 Ozone generating sterilizer (DEL industries) and monitored with a Dasibi 1003-AH ozone analyzer (Dasibi Environmental Corp.). Ozone-fumigation (500 nL L^{-1}) of poplar plants was carried out by placing the plants into the above-mentioned gassing chamber for 30 min and 12 hr, followed by freezing leaf tissue in liquid nitrogen and -80°C storage.

Plant transformation and PCR analysis

Agrobacterium tumefaciens harboring the recombinant binary vector pBIN 19/pRT 101- SIPK (Samuel and Ellis 2002) was used for plant transformation. A published method for *A. tumefaciens*-mediated transformation (AT) of poplar hybrid 717 (Leple *et al*. 1992) was employed. After cocultivation with *A. tumefaciens*, leaf discs were first cultured on MS medium without any antibiotics for two days and then transferred onto MS medium containing 500 mg/L cefotaxime and 100 mg/L kanamycin for the selection of transformants. Putative SIPK- poplar transgenics were detected through PCR using a forward primer specific to the CaMV 35S promoter sequence (ATGACGCACAATCCCACT) together with a gene-specific reverse primer, (5'-CGCTCGAGATTCACATAT GCTGGTATTCAGGATTAAATGC-3'). The PCR-positive transgenic poplar plantlets were subsequently transferred to soil and grown under growth chamber conditions (see above). Positives were then also screened through Western blot analysis (see below) using an anti-FLAG antibody.

Protein extraction

The frozen tissue was ground in liquid nitrogen and the powder stirred with 2 vol of extraction buffer (50 mM Hepes pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM PMSF, 2 µg/ml antipain, 2 µg/ml leupeptin, 10 µg/ml aprotinin, 5 µg/ml pepstatin, glycerol (10%), polyvinylpolypyrrolidone (7.5%)) was added and kept on a reciprocating shaker at (100 oscillations/minute) for 10 min, at 4°C, followed by centrifugation at 15,000 rpm for 30 min. The supernatant was assayed directly or flash-frozen and stored at -80°C.

Western blot analysis

A characteristic of MAPKs is that they posses a conserved phosphorylation motif within the catalytic domain. The two sites of phosphorylation reside on a phosphopeptide separated by only one residue (**-T**x**Y-**). Antibodies raised against mammalian protein kinases (ERK1&2) have been successfully used to detect the presence of homologous proteins in plants (Wilson *et al*. 1995; Knetsch *et al*. 1996; Miles *et al*. 2002). Antibodies raised against the phosphorylated form of the MAPK will only recognize the doubly phosphorylated form and not the unphosphorylated form of these MAPKs. Since the anti-pERK 1&2 antibody only detects the active (phosphorylated) form and not the inactive (unphosphorylated) form, it becomes a powerful tool for monitoring the activation of ERK1&2 homologues in plants (Samuel *et al*. 2000; Miles *et al*. 2002, 2005).

The protein content was quantified using the Bradford dye-binding assay. Extracted proteins (30 µg) for poplar leaf tissue, were fractionated in a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and transferred onto polyvinylidenedifluoride (PVDF) membranes (Millipore, Nepean, Ontario, Canada). The PVDF membranes were then probed with anti-pERK 1&2 antibodies (New England Biolabs Inc., Beverly, MA, USA) or anti-FLAG antibodies (Sigma). Peroxidase-conjugated goat anti-rabbit IgG for the phospho-specific antibodies and goat anti-mouse IgG for the anti-FLAG antibody (Dako Corp., Carpinteria, CA, USA) were used as the secondary antibodies. A primary antibody dilution of 1:1000 was used for the anti-pERK 1&2 antibodies, and 1:5000 was used for anti-FLAG. Both secondary antibodies were used at a dilution of 1:8000. The resulting immune complexes were then visualized using the enhanced chemiluminescence detection kit (Amersham Corp., Baie d'Urfe, Quebec, Canada) following the manufacturer's specifications. All experiments were completed in triplicate.

In situ staining for hydrogen peroxide

H_2O_2 accumulation was detected *in situ* by 3, 3'-diaminobenzidine staining (Torres *et al*. 2002; Miles *et al*. 2005). Ozone-exposed (500 nL L^{-1}, 12 hr) leaves were vacuum infiltrated with the 3, 3'-diaminobenzidine (DAB) (1 mg/ml) solution. Infiltrated leaves were placed under high humidity until brown precipitation was observed (5 to 6 hr) and then fixed with a solution of ethanol: lactic acid: glycerol (3: 1: 1, v/v) for 2 days, followed by further clearing in methanol.

RESULTS AND DISCUSSION

Identification of transgenic poplar lines overexpressing SIPK

The ROS-induced MAPK signalling components appear to be broadly functional across plant taxa. The Arabidopsis MAPKKs, MKK4 and MKK5, have been shown to be functional in tobacco (Ren *et al*. 2002). Shou *et al*. (2004) demonstrated that expression of NPK1, a tobacco MAPKKK, in maize leads to enhanced freezing tolerance, and the tobacco NQK MAPK matrix components were also shown to complement the yeast mutants (Soyano *et al*. 2003). Both SIPK and MPK6 are known to control oxidant signalling in tobacco and Arabidopsis, respectively (Kovtun *et al*. 2000; Samuel and Ellis 2002; Miles *et al*. 2005). And most recently, MKK5 has been shown to be involved in ozone-induced activation of both MPK3 and MPK6 (Miles *et al*. 2009b).

To explore this possibility in tree species, SIPK-FLAG construct was ectopically overexpressed in poplar, and these transgenics were assessed for their altered sensitivity to ozone. A number of transgenic poplar lines that stably overexpressed SIPK-FLAG to various levels (**Fig. 1A**) were identified. When representative lines were tested for the activation of SIPK by ozone, strong activation of SIPK was observed 30 min following ozone exposure along with the activation of endogenous MAPKs (**Fig. 1B**). The ozone-induced activation of the endogenous MAPKs, measured by

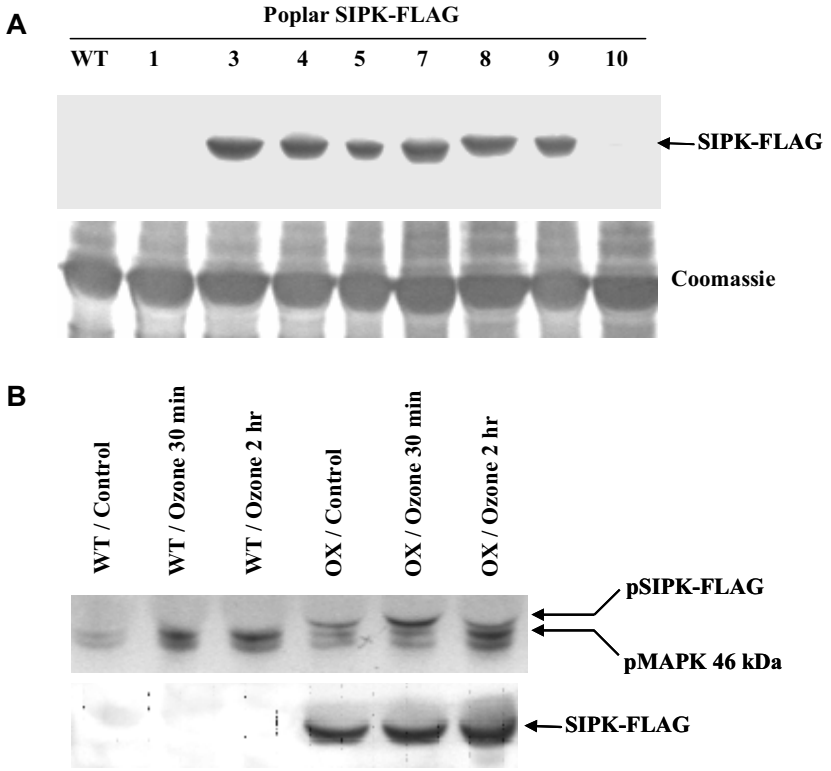

Fig. 1 SIPK activity is inducible in transgenic poplar plants overexpressing SIPK-FLAG. Proteins (30 μg) extracted from leaves of the different SIPK-FLAG overexpressing (OX) lines were immunoblotted using anti-FLAG antibody (**A-**top). The blot was stained with coomassie dye to show equal loading (**A-**bottom). Crude protein extracts prepared from ozone-exposed tissues from T_1 transgenic poplar seedlings from line OX4 and the WT were resolved in a 10% polyacrylamide gel, blotted, and probed with an anti-phospho-ERK antibody to recognize phospho-MAPK forms (**B-**top). The overexpression of SIPK-FLAG in the transgenic line was confirmed by probing the same blot with anti-FLAG antibody (**B-**bottom).

band intensity, was found to be consistently delayed and less intense in the SIPK overexpressing lines compared to that of the WT lines. Interestingly, SIPK was activated earlier than the endogenous MAPKs, but was back to near background within two hours. This transient activation pattern was dependent on the phosphorylation status since the total protein levels of SIPK-FLAG did not change during the course of the treatment (**Fig. 1B**, bottom). Similar results have been observed previously in tobacco plants overexpressing SIPK (Samuel and Ellis 2002). This effect is most likely due to saturation of the system with the ectopically expressed kinase which competes effectively for upstream activators.

SIPK overexpression alters sensitivity of poplar plants to ozone

When these transgenic poplar lines were tested for their sensitivity to ozone, the SIPK overexpressing lines (OX4 and OX5), which were the central parental lines for this study, displayed increased sensitivity to ozone compared to WT plants, which showed no detectable damage (**Fig. 2A**). Lesions appeared consistently 12 hr after ozone exposure along with concomitant accumulation of H_2O_2, as detected by 3, 3'-diaminobenzidine (DAB) staining around the lesions in the transgenic lines, while WT lines lacked lesions and H_2O_2 (**Fig. 2B, 2C**). In tobacco, overexpression of SIPK resulted in increased sensitivity of plants to ozone stress and heightened ozone-induced accumulation of H_2O_2 (Samuel and Ellis 2002). We next wanted to evaluate the SIPK overexpressing lines to a protracted exposure episode. Transgenic poplar lines (OX4 and OX5), along with WT plants, were exposed to ozone (500 nL L^{-1}) for 24 hr followed by three days in an ozone-free environment (recovery chamber) and evaluated for gross ozone-induced damage (**Fig. 3**). There was no detectable leaf damage on WT leaves, whereas both transgenic lines, especially OX4, showed extensive ozone-induced flecking, a cell death response characteristic to ozone damage.

The observed phenotype in poplar essentially mimicked our observations with tobacco plants overexpressing SIPK (Samuel and Ellis 2002). The functional similarity of SIPK in both tobacco and poplar provides strong evidence for a conserved MAPK module that serves as central oxidant-induced signal transducer in both herbaceous and tree species. Manipulation of this MAPK module should provide avenues to improve and create pollutant-tolerant poplar lines. Alternatively, these SIPK-overexpressing poplar lines could be used as biomarkers for increased ozone pollution providing an effective strategy to monitor ozone levels in polluted air.

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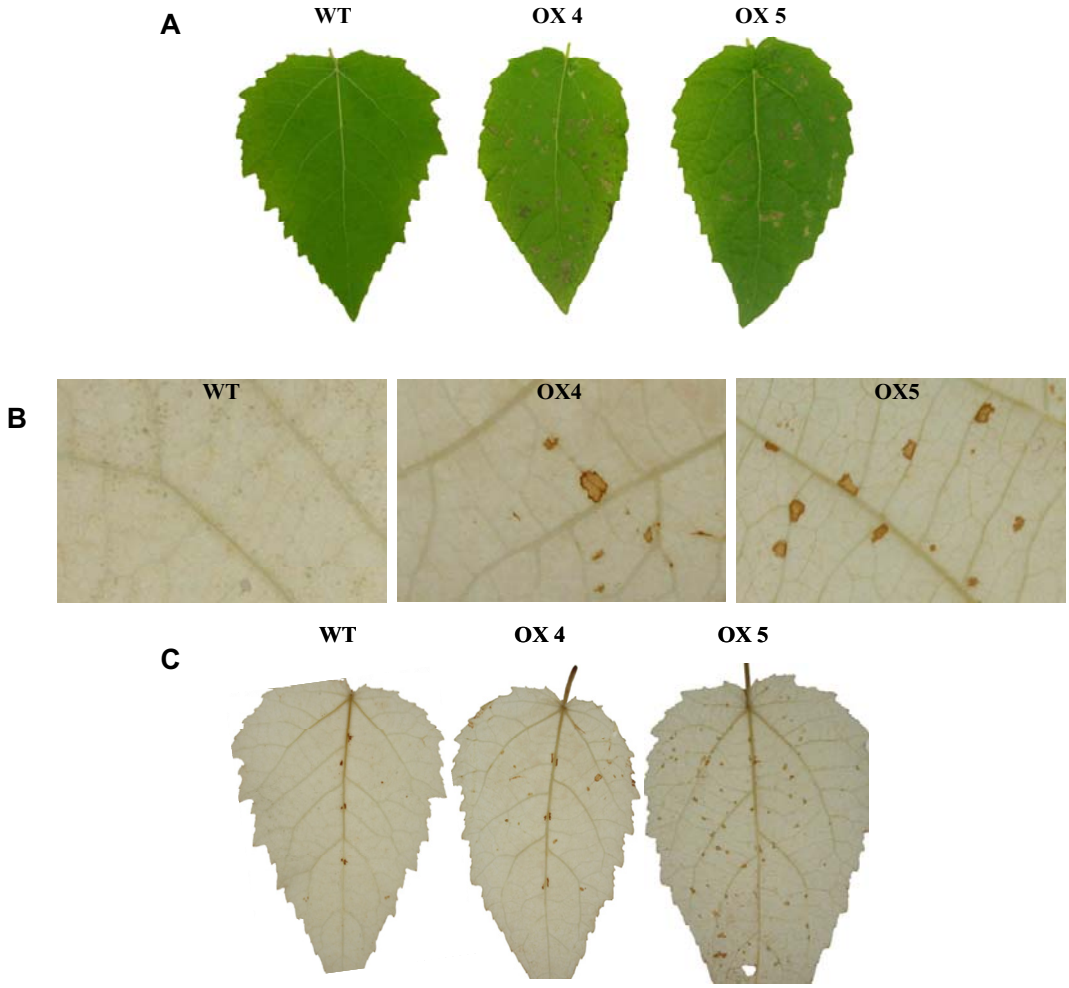

Fig. 2 SIPK overexpressing transgenic poplar plants are hypersensitive to ozone exposure. Transgenic poplar lines OX4, OX5 and WT plants were exposed to ozone (500 nL L^{-1}) for 12 hr. The treated leaves were photographed 24 hr after exposure (**A**). Following 12 hr of ozone exposure, 3, 3'-diaminobenzidine (DAB) staining was used to detect H_2O_2 accumulation (close-up view) in ozone-treated leaves of transgenic and WT plants (**B**) and a full-leaf view of these same DAB treated leaves (**C**).

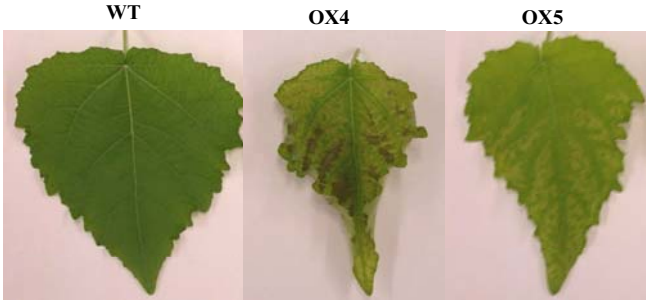

Fig. 3 SIPK overexpressing transgenic poplar plants exposed to ozone for extended duration. Transgenic poplar lines OX4, OX5 and WT plants were exposed to ozone (500 nL L^{-1}) for 24 hr and photographed 72 hr later. There was no detectable leaf damage on the WT leaves, whereas both transgenic lines, especially OX4, showed extensive ozone-induced leaf damage.

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