

Endogenous overexpression of *Populus MYB186* increases trichome density, improves insect pest resistance, and impacts plant growth

Jonathan M. Plett¹, Olivia Wilkins², Malcolm M. Campbell^{2,3,4}, Steven G. Ralph⁵ and Sharon Regan^{1,*}

¹Department of Biology, Queen's University, Kingston, ON K7L 3N6, Canada,

²Department of Cell & Systems Biology, University of Toronto, 25 Willcocks St., Toronto, ON M5S 3B2, Canada,

³Department of Biology, University of Toronto Scarborough, 1265 Military Trail, Toronto, ON M1C 1A4, Canada,

⁴Centre for the Analysis of Genome Evolution and Function, University of Toronto, 25 Willcocks Street, Toronto, ON M5S 3B2, Canada, and

⁵Department of Biology, University of North Dakota, Grand Forks, ND 58202-9019, USA

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*For correspondence (fax +613 533 6617; e-mail sharon.regan@queensu.ca).

SUMMARY

Trichomes are specialized epidermal cells that generally play a role in reducing transpiration and act as a deterrent to herbivory. In a screen of activation-tagged *Populus tremula* × *Populus alba* 717-1B4 trees, we identified a mutant line, *fuzzy*, with increased foliar trichome density. This mutant also had a 35% increase in growth rate and a 200% increase in the rate of photosynthesis as compared with wild-type poplar. The *fuzzy* mutant had significant resistance to feeding by larvae of the white-spotted tussock moth (*Orgyia leucostigma*), a generalist insect pest of poplar trees. The *fuzzy* trichome phenotype is attributable to activation tagging and increased expression of the gene encoding *PtaMYB186*, which is related to *Arabidopsis thaliana* *MYB106*, a known regulator of trichome initiation. The *fuzzy* phenotype can be recapitulated by overexpressing *PtaMYB186* in poplar. *PtaMYB186* overexpression results in reconfiguration of the poplar transcriptome, with changes in the transcript abundance of suites of genes that are related to trichome differentiation. It is notable that a plant with misexpression of a gene responsible for trichome development also had altered traits related to growth rate and pest resistance, suggesting that non-intuitive facets of plant development might be useful targets for plant improvement.

Keywords: poplar, activation tagging, R2R3-MYB, trichome, herbivory, growth rate.

INTRODUCTION

Trichomes are enlarged, modified epidermal cells that grow perpendicular to the leaf surface. As their density increases, trichomes can decrease transpiration rates (Choinski and Wise, 1999; Perez-Estrada *et al.*, 2000) and increase defence against herbivores and parasites (Neal *et al.*, 1989; Bodnaryk, 1996). On the other hand, trichomes may also have a negative impact on plant growth and vigour, as they are costly to produce. Trichomes can decrease the absorption of sunlight, impede transpiration and decrease the rate of carbon dioxide diffusion, thereby interfering with the rate of photosynthesis (Billings and Morris, 1951; Pearman, 1966; Sinclair and Thomas, 1970; Ehleringer *et al.*, 1976; Eller and Willi, 1977), although this is not always the case (Shull, 1929; Gausman and Cardenas, 1969, 1973; Wuenschel, 1970).

Trichome initiation has been well characterized in *Arabidopsis thaliana*, and, to a lesser extent, in other model plant systems. Of the genes that control trichome development, a number have been found to be members of the MYB family of transcription factors (Avila *et al.*, 1993; Noda *et al.*, 1994; Kirik *et al.*, 2001; Ishida *et al.*, 2008; Jakoby *et al.*, 2008; Shangguan *et al.*, 2008). Two notable MYBs that control trichome development are GLABRA1 (GL1; Oppenheimer *et al.*, 1991) and TRYPTICHON (TRY; Schellmann *et al.*, 2002), which competitively initiate or inhibit trichome formation, respectively (Ishida *et al.*, 2008). Other MYB proteins that affect trichome development in both *Arabidopsis* and other species are MYB23 (Kirik *et al.*, 2001), MIXTA (Noda *et al.*, 1994), PhMYB1 (Avila *et al.*, 1993), GaMYB2 and

GaMYB25 (Jakoby *et al.*, 2008; Shangguan *et al.*, 2008). NOECK (NOK) is involved in negatively regulating branching through the repression of ANGUSTIFOLIA in *A. thaliana* trichomes, as loss-of-function mutations to this gene result in trichomes with increased branching (Folkers *et al.*, 1997). Recently, NOK was identified as MYB106, a MIXTA-like transcription factor (Jakoby *et al.*, 2008).

Like the trichomes of *A. thaliana*, poplar trichomes are uni-nucleate, uni-cellular modified epidermal cells. Unlike *A. thaliana*, the trichome density of which is tied to leaf position and plant developmental stage, poplar trichome production does not vary significantly in leaves along a particular shoot axis both when grown in culture or in soil. Little is known about the genetic basis controlling poplar trichome differentiation and development, although homologues of trichome regulatory genes have been identified (Wilkins *et al.*, 2009a,b). It is likely, based on a preliminary examination of the poplar genome, that there are homologous genes between poplar and *A. thaliana* that have different roles in poplar trichome morphogenesis. For example, despite the fact that poplar encodes numerous homologues to genes known to control trichome branching in *A. thaliana* (e.g. *PtaMYB186*), poplar trichomes never undergo branching. Therefore, it is necessary to functionally characterize these genes in poplar, as trichomes are closely tied to deciduous tree defence (Ågren and Schemske, 1993; Mauricio, 1998; Gruber *et al.*, 2006; Kivimäki *et al.*, 2007), transpiration (Benzing, 1976; Martin and Schmitt, 1989; Smith and McClean, 1989; Espigares and Peco, 1995) and photosynthesis (Benzing and Renfrow, 1971; Benzing *et al.*, 1976). Moreover, the pleiotropic impact of the investment in trichome development on aspects of plant growth and development, including growth rate, have not been extensively investigated in tree species. Such impacts could be significant as long-lived arboreal species, like poplar trees, are frequently exposed to multiple challenges by pests and pathogens over their lifetimes, and must balance resource investment into defences such as trichomes against resource availability over the long term.

In an effort to identify new genes involved in the regulation of many facets of growth and development in poplar, a population of 1800 activation-tagged poplars was phenotyped (Harrison *et al.*, 2007). Here, we detail the characterization of one mutant line, *fuzzy*, which had an increase in trichome density. We found that overexpression of the gene *PtaMYB186*, a close homologue of the *A. thaliana* MYB106 gene, accounted for increased trichome density in the *fuzzy* mutant. Consistent with this, we found that overexpression of *PtaMYB186* resulted in changes in transcript abundance of suites of genes involved in trichome formation. To characterize the pleiotropic effects of increased trichome density, *fuzzy* mutants were compared with wild-type poplar plants for differences in transpiration, photosynthesis, growth rate, and resistance to a poplar herbivore, the

white-marked tussock moth (*Orgyia leucostigma*). This study provides insights into the mechanisms underpinning trichome development in a woody perennial plant, and suggests that increased trichome density may aid plant growth while concurrently increasing resistance to herbivory.

RESULTS

In a screen for visible phenotypes in activation-tagged mutant poplars, the *fuzzy* mutant was identified on the basis of an increase in leaf trichome density (Figure 1). Fully expanded wild-type leaves had 41 trichomes cm⁻² on the adaxial surface, whereas *fuzzy* had 7509 trichomes cm⁻² (Table 1). On the abaxial side of the leaf, wild-type leaves had 5688 trichomes cm⁻² compared with 40 015 trichomes cm⁻² on *fuzzy* (Table 1). Abaxial stomata and epidermal cell densities were not significantly different between wild-type and the *fuzzy* mutant, therefore the observed increase in trichome density is not the result of altered pavement cell expansion or division (Table 1). Also, there was no significant difference in the length of trichomes on wild-type and *fuzzy* leaves. Freeze-fracture microscopy of *fuzzy* leaves revealed that the increased number of trichomes produced a thicker, taller trichome canopy on the abaxial side of the leaf (Figure 1d,f).

As reported previously (Harrison *et al.*, 2007), the *fuzzy* mutant had only one insertion event of the activation tag T-DNA. The insertion site was mapped to chromosome 8 at position 553 000 using a modified thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) strategy. Predicted gene models within 10 kb upstream and downstream of the insertion site included *PtaMYB186*, a putative E3 ubiquitin ligase (protein ID 765763) and a putative ATP/GTP-binding protein (protein ID 765765).

To identify the activated gene(s) in the vicinity of the T-DNA insertion, we measured transcript abundance in the shoot apex using real-time quantitative PCR, as the cellular mechanisms responsible for trichome initiation are most active in the young developing leaves. In the shoot apices of *fuzzy* mutants *PtaMYB186* had eightfold greater transcript abundance compared with the same tissue in wild-type trees, whereas transcripts for the other two genes were below the limit of detection (Figure 2a). The increased transcript abundance of *PtaMYB186* in *fuzzy* mutants relative to wild-type poplar was also confirmed using microarray-based whole transcriptome analysis on the same tissue. Transcript levels in different aerial tissues of wild-type poplar clone 717-1B4 indicates that *PtaMYB186* is normally expressed in the shoot apex and the epidermis of young stems, whereas transcript levels were below the detection limit in mature leaves and other tissues of the stem (data not shown).

To determine if ectopic overexpression of *PtaMYB186* was responsible for the *fuzzy* phenotype, the gene was

Figure 1. *fuzzy* mutant shows increased density of trichomes on leaf surfaces. Wild-type (a) and *fuzzy* (b) shoot apex. Abaxial side of a fully expanded wild-type (c) and *fuzzy* (e) leaf; scale bars = 4 cm. Scanning electron microscopy image of abaxial side of fully expanded wild-type leaf (d) and *fuzzy* (f) leaf; scale bars = 40 μm . Freeze fractured leaf cross section of wild type (g) and *fuzzy* (h) showing the trichome canopy extending downwards from the abaxial side of the leaf; scale bars = 100 μm .

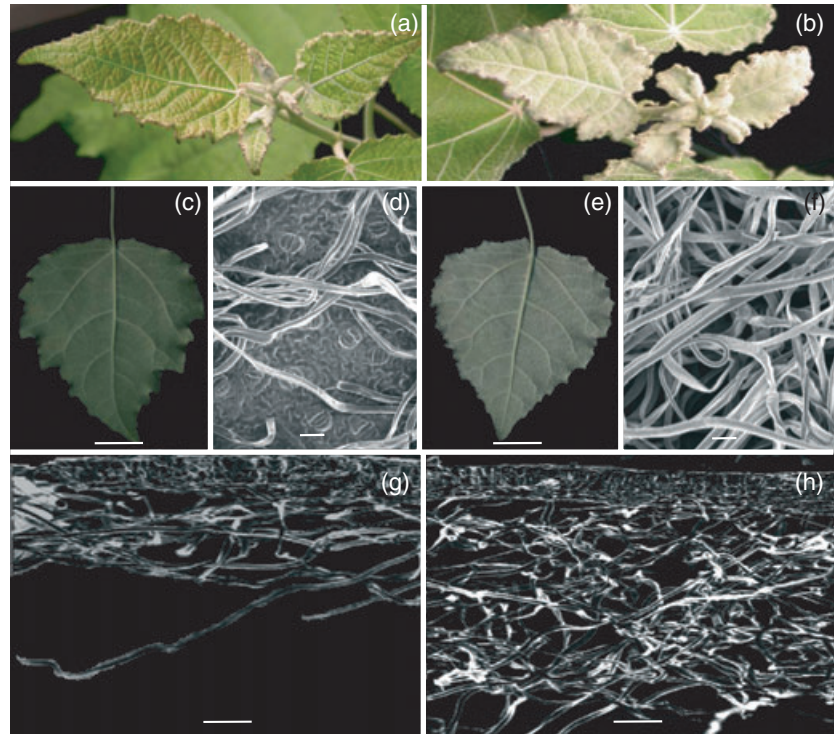


Table 1 Physiological characteristics of wild-type and *fuzzy* poplar lines

	717	n	<i>fuzzy</i>	n
Adaxial trichomes (Trichome cm^{-2})	41 \pm 16	16	750* \pm 160	15
Adaxial trichome length (μm)	390 \pm 17	30	424 \pm 30	30
Abaxial trichomes (Trichome cm^{-2})	5688 \pm 2183	30	40 015* \pm 9731	28
Abaxial stomates (Stomates cm^{-2})	31 238 \pm 5926	30	31 004 \pm 5111	28
Abaxial epidermal cells (Cell cm^{-2})	241 179 \pm 4315	19	250 640 \pm 4482	15
Leaf area (cm^2)	153 \pm 28	30	183* \pm 20	31
Leaf dry weight (%)	47 \pm 5	31	34 \pm 1.4	30
Internode length (cm)	30.6 \pm 1.3	10	32.6 \pm 1.3	10
Transpiration Rate ($\text{mol m}^{-2} \text{s}^{-1}$)	0.001 \pm 0.0007	20	0.002* \pm 0.0002	22
CO ₂ exchange rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	3.84 \pm 1.2	20	8.23* \pm 2	22
Total conductance to H ₂ O ($\text{mol m}^{-2} \text{s}^{-1}$)	0.06 \pm 0.02	20	0.11* \pm 0.02	22
Total resistance to H ₂ O ($\text{m}^2 \text{s mol}^{-1}$)	18.8 \pm 4.7	20	9.35* \pm 1.15	22
Total conductance to CO ₂ ($\text{mol m}^{-2} \text{s}^{-1}$)	0.04 \pm 0.01	20	0.07* \pm 0.01	22
Total resistance to CO ₂ ($\text{m}^2 \text{s mol}^{-1}$)	30.4 \pm 7.6	20	15.05* \pm 1.9	22
Water use efficiency	3.98 \pm 1.00	20	3.43 \pm 1.39	22

introduced into wild-type clone 717-1B4 *Populus tremula* \times *Populus alba* under the control of the 35S CaMV promoter. The young leaves of wild-type poplars grown in tissue culture have very few abaxial trichomes (Figure 2b), whereas *fuzzy* plants have a much denser array of trichomes (Figure 2c). Compared with other poplar transformation experiments that we have carried out, the frequency of transformed plants was much lower with this particular construct, and multiple transformation experiments were required to produce the transformed lines described here (data not shown), plus several of the putative lines were not viable out of tissue culture. In our experience, this suggests that activation of this gene with the 35S promoter is unfavourable to plant regeneration.

Of the six lines recovered from the transformation of 35S::*PtaMYB186* into wild-type poplar, two lines had more trichomes than either the wild type or *fuzzy* (Figure 2d,e), three lines had trichome densities similar to *fuzzy* (Figure 2f–h) and one 35S::*PtaMYB186* line had wild-type levels of trichome initiation in tissue culture (Figure 2i). Of the six lines recovered by transformation, four of the lines were not able to be successfully transferred to soil. Of the two lines that were transferred to soil (lines 1 and 3), both had increased trichome initiation on leaves (Figure 2l,m) and a fourfold and 39-fold greater transcript abundance of *PtaMYB186* in comparison with the wild type, respectively. Taken together, these findings support the hypothesis that ectopic overexpression of *PtaMYB186*

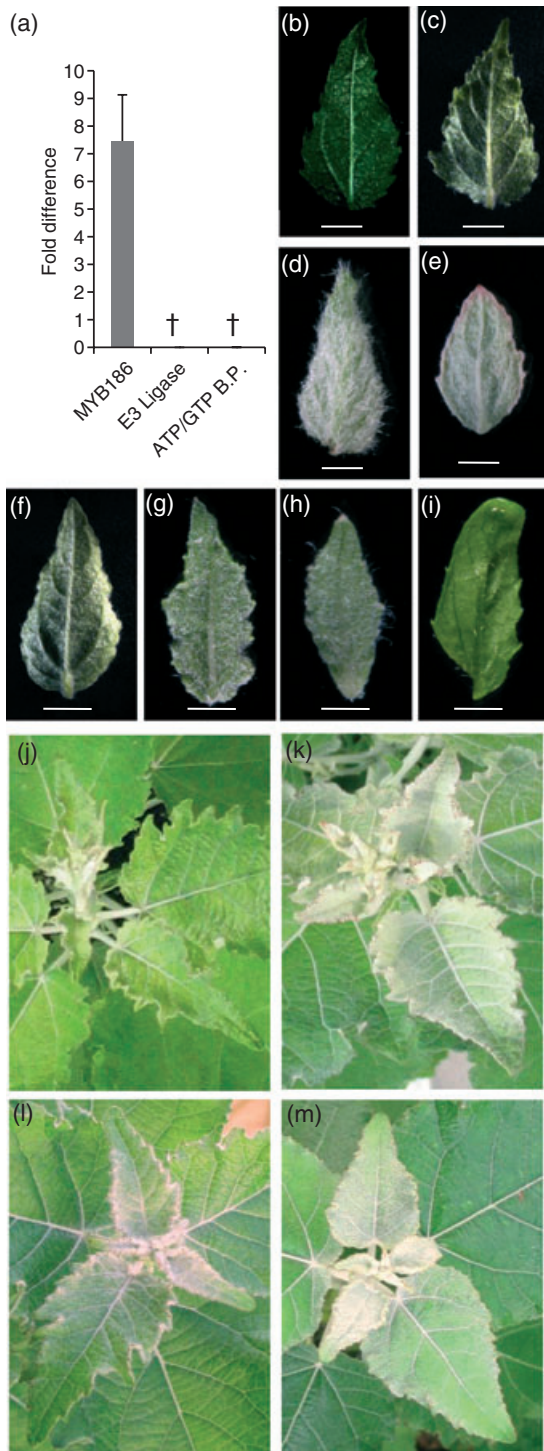


Figure 2. *PtaMYB186* is overexpressed in the *fuzzy* mutant and is responsible for the trichome phenotype. (a) Overexpression of *PtaMYB186* was detected using real-time PCR in the shoot apex of *fuzzy* lines, relative to transcript levels in wild-type shoot apex. †Expression of two other genes near the insertion site of the activation tagging T-DNA were below detection levels in both wild-type and *fuzzy* lines. Abaxial side of a newly produced wild-type leaf grown in tissue culture (b), of *fuzzy* (c) and of the six independent transformants of *35S::PtaMYB186* recovered (lines 1–6; d–i). Shoot apices of soil-grown wild-type poplar (j), *fuzzy* (k), *35S::PtaMYB186* line 1 (l) and *35S::PtaMYB186* line 3 (m). Scale bars for b–i = 3 mm.

increased trichome initiation and probably accounted for the *fuzzy* phenotype.

Transcriptome analysis of the shoot apices of *fuzzy* and wild-type plants was undertaken to examine the molecular underpinnings of the *fuzzy* phenotype. This analysis found that the *PtaMYB186* transcript (PtpAffx.85736.1.S1) was at significantly higher levels in the shoot apices of *fuzzy* than in the apices of wild-type plants ($\log_2FC = 0.95$, one-sided Student's *t*-test, $t = -5.8$, d.f. = 4, $P = 0.002$). Additionally, a further 753 genes were identified that were characterized by either higher or lower levels of transcript accumulation in *fuzzy* compared with wild-type plants (Figure S1; Tables S1 and S2). The 35 genes with the largest differences in transcript abundance between *fuzzy* and wild-type plants are listed in Tables 2 and 3. Strikingly, of the 753 genes with significant differences in transcript abundance, the majority (716) had lower transcript abundance in *fuzzy* mutants in comparison with wild type (Figure S1; Tables S1 and S2).

A comparison of the GOslim annotations of the genes with differences in transcript abundance between *fuzzy* and wild-type shoots is presented in Figure 3. Notably, the poplar homologues of genes implicated in trichome development in *A. thaliana*, including *KAKTUS*, *TRANSPARENT TESTA GLABRA 2*, *CALOSE SYNTHASE 1* and *CYCLIN DEPENDENT KINASE C1*, were prominent amongst the genes with decreased transcript abundance in *fuzzy* mutants relative to wild-type plants. There were also a number of different gene families that were over-represented amongst the genes with decreased transcript abundance. These included genes implicated in reproduction or circadian rhythm (i.e. *FY*, *LHY*), defence-related compounds (i.e. *TT7*, *PRL1*), cell wall biosynthesis and cell division (i.e. *CYC3B*, *MRH5*), chloroplast/photosynthetic genes (i.e. *AtGLDP2*, *SIGB*) and hormone signalling (i.e. *EIN3*, *ERS1*; Figure 3).

Of the 35 genes with increased transcript abundance in *fuzzy* mutants relative to wild-type plants, 16 encoded proteins with predicted roles in protein turnover, including a number of heat-shock proteins (Table S2). Recent evidence suggests that protein turnover is a feature of trichome differentiation, as these same genes have been found to have increased transcript abundance in developing Arabidopsis trichomes (Jakoby *et al.*, 2008; Lieckfeldt *et al.*, 2008; Marks *et al.*, 2009). Additional genes also implicated in trichome development that also had significant increases in transcript abundance included *PtaMYB186*, and the genes homologous to Arabidopsis *MYB4* and *SHINE3* (*SHN3*).

To determine if the increase in trichome density on the adaxial surface of the leaf affected light absorbance, and thus by extension photosynthetic potential, leaf plastochron index 12–42 on *fuzzy* and wild-type trees were analysed using the normalized difference vegetation index (NDVI) (Figure 4). Healthy leaves absorbing normal levels of light have an NDVI value of 0.8–0.9. Conversely, foliage that does not absorb an optimal level of light (e.g. as a result of

Table 2 Thirty five differentially expressed genes with the greatest increase in abundance in wild-type shoot apices relative to the apices of *fuzzy* mutant with GOslim annotation

Poplar gene model	Fold change (Log2)	AGI homologue	<i>Arabidopsis thaliana</i> homologue annotation	P
fgenes4_pg.C_LG_XVI000099	8.97	AT4G01985	Unknown protein	0.00012
gw1.l.3053.1	8.30	ATCG00270	Unknown protein	0.00000
estExt_fgenes4_pg.C_1520062	8.09	AT1G15690	AVP1 (vacuolar-type H ⁺ -pumping pyrophosphatase 1)	0.00277
fgenes4_pm.C_scaffold_376000002	7.88	ATCG00270	Unknown protein	0.00000
fgenes4_pg.C_LG_I002818	7.83	AT1G21680	Unknown protein	0.00000
gw1.44.412.1	7.60	ATCG00680	Unknown protein	0.00170
estExt_fgenes4_pg.C_LG_I0019	7.58	AT3G13460	ECT2	0.00032
gw1.422.10.1	7.55	ATCG00280	Unknown protein	0.00006
grail3.0376000701	7.46	ATCG00680	Unknown protein	0.00000
eugene3.00181092	7.37	AT1G15690	AVP1 (vacuolar-type H ⁺ -pumping pyrophosphatase 1)	0.00261
grail3.0074003701	7.09	AT5G65970	MLO10 (MILDEW RESISTANCE LOCUS O 10)	0.00000
grail3.0074003801	7.09	AT3G51950	Zinc finger (CCCH-type) family protein	0.00000
grail3.0220000401	7.09	AT5G12440	Zinc finger (CCCH-type) family protein	0.00000
gw1.1l.3698.1	6.98	ATCG00670	Unknown protein	0.00056
estExt_fgenes4_pg.C_LG_II2062	6.95	AT2G05710	Aconitate hydratase	0.00000
fgenes4_pg.C_scaffold_15705000001	6.91	AT4G24690	Ubiquitin-associated (UBA)/TS-N domain-containing protein	0.00075
estExt_fgenes4_pm.C_880008	6.87	AT4G13930	SHM4 (SERINE HYDROXYMETHYLTRANSFERASE 4)	0.00190
estExt_fgenes4_pg.C_LG_XVIII0149	6.85	AT2G25970	KH domain-containing protein	0.00038
estExt_Genewise1_v1.C_LG_XIII1701	6.82	AT3G04470	Unknown protein	0.00102
estExt_fgenes4_pm.C_LG_XIV0527	6.65	AT1G05160	CYP88A3 (ENT-KAURENOIC ACID HYDROXYLASE 1)	0.00000
gw1.131.1.1	6.61	AT5G26742	EMB1138 (EMBRYO DEFECTIVE 1138)	0.00190
gw1.VIII.2633.1	6.61	AT4G37930	SHM1 (SERINE HYDROXYMETHYLTRANSFERASE 1)	0.00211
eugene3.00051523	6.59	AT1G76160	SKS5 (SKU5 Similar 5)	0.00136
eugene3.10060002	6.47	AT1G30330	ARF6 (AUXIN RESPONSE FACTOR 6)	0.00000
gw1.XIII.3135.1	6.39	AT1G33470	RNA recognition motif (RRM)-containing protein	0.00236
gw1.XIV.449.1	6.37	AT5G65620	peptidase M3 family protein	0.00006
eugene3.30290001	6.28	ATCG00170	Unknown protein	0.00043
eugene3.00190393	6.27	AT1G55500	ECT4	0.00022
estExt_Genewise1_v1.C_LG_XIV1619	6.26	AT4G01070	GT72B1	0.00245
eugene3.01180054	6.24	AT4G18030	Dehydration-responsive family protein	0.00186
estExt_fgenes4_pm.C_LG_XVIII0347	6.21	AT5G57940	ATCNG5 (CYCLIC NUCLEOTIDE GATED CHANNEL 5)	0.00002
estExt_fgenes4_pg.C_1470038	6.20	AT2G24520	AHA5 (ARABIDOPSIS H(+)-ATPASE 5)	0.00000
grail3.0013021201	6.18	AT2G25970	KH domain-containing protein	0.00002
grail3.0050011001	6.18	AT3G62360	Carbohydrate binding	0.00002
eugene3.30290002	6.17	ATCG00190	Unknown protein	0.00000

barriers such as trichomes) will have a much lower NDVI value (Sellers, 1985; Myneni *et al.*, 1995). NDVI values for both the *fuzzy* mutant and the wild type were not significantly different for the young, photosynthetically active leaves (12–32). Leaves 33–42 of the wild type, however, had significantly lower NDVI values ($P < 0.05$). Taken together, these data indicated that the increases in trichome density on the adaxial surface of *fuzzy* leaves do not significantly decrease the quantity of light absorbed by *fuzzy* leaves, and would thus suggest that *fuzzy* trichome density is not hindering photosynthesis. Additionally, it is interesting to note that the older leaves of *fuzzy* (leaves 33–42) absorb light more optimally than do wild-type plants, although this small difference is unlikely to have a significant impact on photosynthesis because of the position of these leaves on the stem.

To evaluate whether the increase in trichomes was correlated with a change in the growth rate of the tree, *fuzzy* and wild-type growth were compared in the glasshouse under natural light conditions (Figure 5a–c). Although the initial growth rate of the mutant after bud break was not significantly different from that of the wild type, the mutant line attained a height of 1.5 m 22 days faster than the wild type (Figure 5b). There was no difference in the internode length between the wild type and *fuzzy* (data not shown). Stem girth also increased faster in the mutant as compared with the wild type (Figure 5c). As increased growth would place an increased demand on the photosynthetic capacity of the tree, we tested the rate of transpiration and photosynthesis of the mutant and the wild type. The mutant had significantly higher rates of transpiration and photosynthesis, although the efficiency of water use in the mutant was

Table 3 Thirty-two differentially expressed genes with the greatest reduction in abundance in the shoot apices of wild-type plants as compared with the apices of *fuzzy* mutants classified according to GOslim annotation

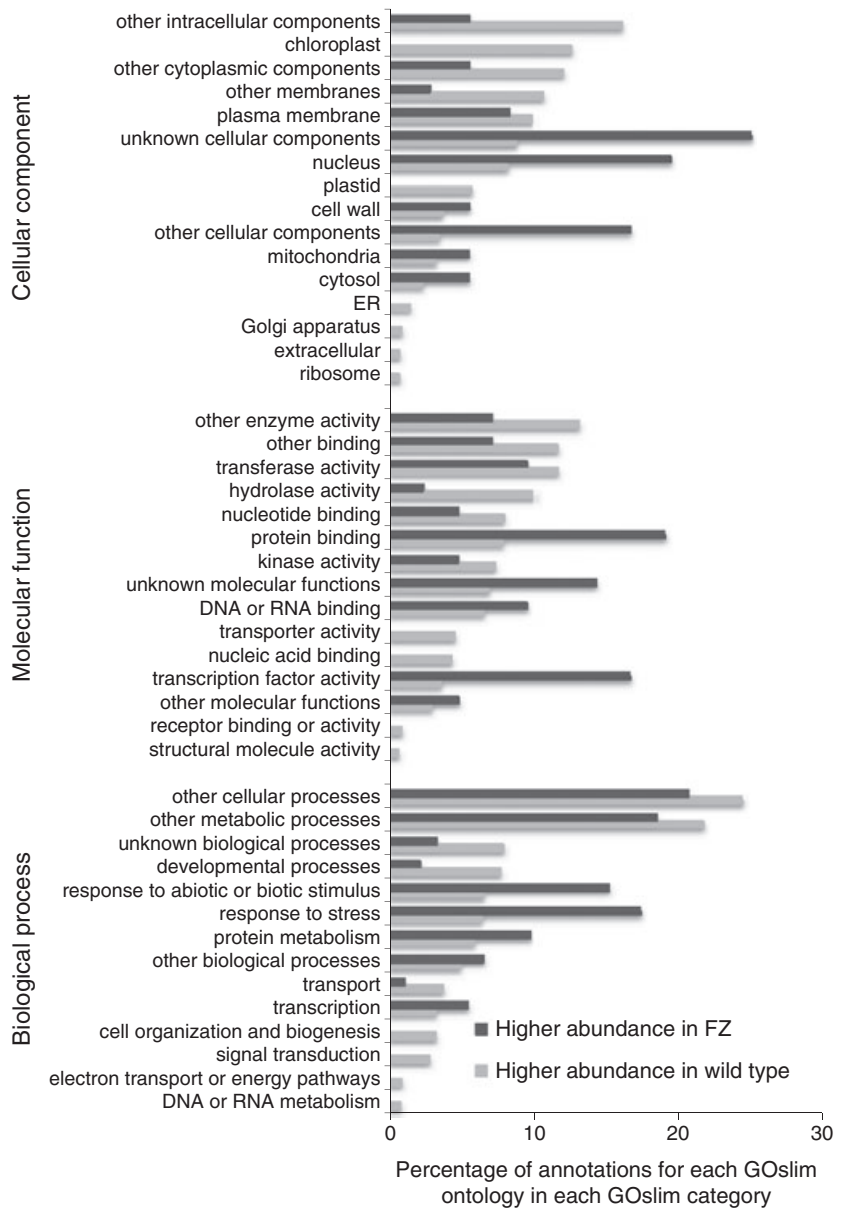
Poplar gene model	Fold change (Log2)	AGI homologue	<i>Arabidopsis thaliana</i> homologue annotation	P
estExt_fgenesh4_pg.C_LG_X1023	-4.50	AT1G56300	DNAJ heat-shock protein	0.00000
eugene3.00131208	-4.33	AT5G60490	FLA12 (fasciclin-like arabinogalactan-protein 12)	0.00003
grail3.0001103101	-3.98	AT2G29500	17.6-kDa class-I small heat-shock protein (HSP17.6B-CI)	0.00003
eugene3.00031372	-3.57	AT1G12060	ATBAG5 (ARABIDOPSIS THALIANA BCL-2-ASSOCIATED ATHANOGENE 5)	0.00004
fgenesh4_pg.C_LG_II001519	-3.49	AT2G46130	WRKY43 (WRKY DNA-binding protein 43)	0.00141
fgenesh4_pg.C_LG_V001233	-3.22	AT2G30766	Unknown protein	0.00000
estExt_Genewise1_v1.C_LG_IX3637	-2.95	AT2G29500	17.6-kDa class-I small heat-shock protein (HSP17.6B-CI)	0.00147
gw1.V.991.1	-2.94	AT3G22830	AT-HSFA6B (<i>Arabidopsis thaliana</i> heat-shock transcription factor A6B)	0.00009
estExt_Genewise1_v1.C_LG_IX0700	-2.86	AT2G29500	17.6-kDa class-I small heat-shock protein (HSP17.6B-CI)	0.00055
gw1.II.950.1	-2.85	AT4G00880	Auxin-responsive family protein	0.00138
grail3.0204000201	-2.83	AT4G18170	WRKY28 (WRKY DNA-binding protein 28)	0.00250
gw1.XVIII.1599.1	-2.82	AT5G11190	SHN3 (SHINE3)	0.00098
estExt_Genewise1_v1.C_LG_X0543	-2.81	AT3G16920	Chitinase	0.00071
grail3.0017034001	-2.75	AT5G56030	HSP81-2 (EARLY-RESPONSIVE TO DEHYDRATION 8)	0.00006
estExt_Genewise1_v1.C_LG_IX0700	-2.72	AT2G29500	17.6-kDa class-I small heat-shock protein (HSP17.6B-CI)	0.00014
eugene3.00190185	-2.67	AT3G04530	PPCK2 (PHOSPHOENOLPYRUVATE CARBOXYLASE KINASE 2)	0.00166
estExt_fgenesh4_pg.C_LG_XIV0888	-2.60	AT5G48570	Peptidyl-prolyl cis-trans isomerase	0.00008
estExt_fgenesh4_pm.C_LG_VIII0711	-2.51	AT3G23410	Alcohol oxidase-related	0.00014
eugene3.00150664	-2.51	AT5G50260	Cysteine proteinase	0.00104
gw1.VIII.1750.1	-2.49	AT1G14870	Unknown protein	0.00222
estExt_Genewise1_v1.C_570289	-2.42	AT1G15380	Lactoylglutathione lyase family protein/glyoxalase I family protein	0.00145
eugene3.00010940	-2.38	AT4G11660	AT-HSFB2B (<i>Arabidopsis thaliana</i> heat-shock transcription factor B2B)	0.00008
estExt_Genewise1_v1.C_LG_III1704	-2.31	AT1G54050	17.4-kDa class-III heat-shock protein (HSP17.4-CIII)	0.00009
gw1.XI.3394.1	-2.29	AT2G31090	Unknown protein	0.00293
eugene3.01240087	-2.25	AT2G20560	DNAJ heat-shock family protein	0.00113
fgenesh4_pm.C_scaffold_187000008	-2.24	AT5G54010	Glycosyltransferase family protein	0.00141
fgenesh4_pg.C_LG_VI001544	-2.17	AT2G26150	ATHSFA2 (<i>Arabidopsis thaliana</i> heat-shock transcription factor A2)	0.00035
estExt_fgenesh4_pg.C_LG_IV1453	-2.15	AT4G38620	MYB4 (myb domain protein 4)	0.00110
fgenesh4_pm.C_LG_IX000642	-2.10	AT2G18370	Protease inhibitor/lipid transfer protein (LTP) family protein	0.00306
eugene3.00640219	-2.02	AT2G20560	DNAJ heat-shock family protein	0.00041
eugene3.00440247	-1.88	AT5G52640	HSP81-1 (HEAT-SHOCK PROTEIN 81-1)	0.00299
eugene3.00002208	-1.79	AT4G02890	UBQ14 (ubiquitin 14)	0.00237

not significantly different from that of the wild type (Table 1; $P < 0.05$).

An increased density of trichomes has been associated with decreased herbivory in different plant models (Ågren and Schemske, 1993; Mauricio, 1998; Gruber *et al.*, 2006; Kivimäki *et al.*, 2007). We conducted both insect development and feeding bioassays to determine if *fuzzy* was more resistant to white-marked tussock moth larvae, a generalist defoliator of several hardwood species, including poplar. In the first bioassay, we monitored larval development using recently hatched first-instar larvae that were fed leaf discs from either wild-type or *fuzzy* trees for 2 weeks, after which their development and gain in mass was assayed (Figure 6A,B).

It was hypothesized that increased trichome number would deter feeding on *fuzzy*, and that the larvae would develop more slowly and generally be smaller in size. Larvae raised on wild-type leaf discs in Petri dishes developed through to fourth or fifth instar and gained, on average, 20.9 mg in fresh weight. Conversely, larvae reared on a diet of *fuzzy* leaf discs only developed to the third or fourth larval instar, and gained only 5.8 mg, a significant reduction (Figure 6b; $P < 0.05$). In a second test of larval development, second-instar larvae were caged on either wild-type or *fuzzy* trees for 14 days, after which their development and weight gain was assessed (Figure 6b). Similar to the first tests, larvae raised on wild-type trees grew significantly bigger (Figure 6B; $P < 0.05$). Finally, choice of feeding bioassays

Figure 3. GOslim classification of genes with differential transcript accumulation in the *fuzzy* mutant relative to wild-type plants. *Arabidopsis thaliana* homologues of poplar genes with altered transcript abundance in *fuzzy* mutants relative to wild-type plants were identified (Tables S1 and S2), and GOslim classification of the *Populus* genes was based on the functional categorization of the *A. thaliana* homologues (homologues were identified by POPGENIE, Sjodin *et al.*, 2009; GO classification by TAIR, Rhee *et al.*, 2003). For each GOslim ontology (e.g. Cellular Component), the number of annotations to terms in a given GOslim category (e.g. Chloroplast) divided by the total number of annotations to any GOslim term in this ontology was determined; this value is expressed as a percentage. GOslim classification included 41 probe sets with higher levels of transcript accumulation in *fuzzy* than in wild type (dark-grey bars), and 766 probe sets with higher levels of transcript accumulation in wild type than in *fuzzy* (light-grey bars). Full GO classification data is available in Tables S3 and S4.



were conducted to measure leaf consumption after 24 h by third- and fourth-instar larvae reared in Petri dishes when given the choice of eating either wild-type or *fuzzy* leaf discs (Figure 6C,D). Leaves with more damage indicate a feeding preference for that genotype. The larvae fed significantly less on *fuzzy* compared with wild-type leaf discs ($P < 0.05$). Data from these three bioassays combined demonstrate that the larvae of the tussock moth not only prefer consuming wild-type leaves over *fuzzy* leaves, but also that larvae feeding on *fuzzy* trees display substantial developmental retardation.

DISCUSSION

Under field conditions plants are threatened with multiple, often simultaneous, biotic and abiotic stressors that impact their growth. One line of defence in many plants is the

development of trichomes on the leaf surface (Levin, 1973; Ågren and Schemske, 1993). Although trichomes can be a selective advantage during periods of stress, trichomes are considered to be energetically 'costly' to produce (Ågren and Schemske, 1993; Mauricio, 1998), and increases in trichome density have been correlated with reduced growth rate (Gruber *et al.*, 2006). Trichomes have also been shown to decrease the level of incident light reaching the leaf surface (Benzing and Renfrow, 1971; Benzing *et al.*, 1976), reduce transpiration through maintenance of a boundary layer of air next to the leaf surface (Benzing, 1976; Martin and Schmitt, 1989; Smith and McClean, 1989; Espigares and Peco, 1995) and decrease pest foraging (Levin, 1973; Skaltsa *et al.*, 1994; Fordyce and Agrawal, 2001). Therefore, our identification of a poplar mutant with an increase in trichome density that

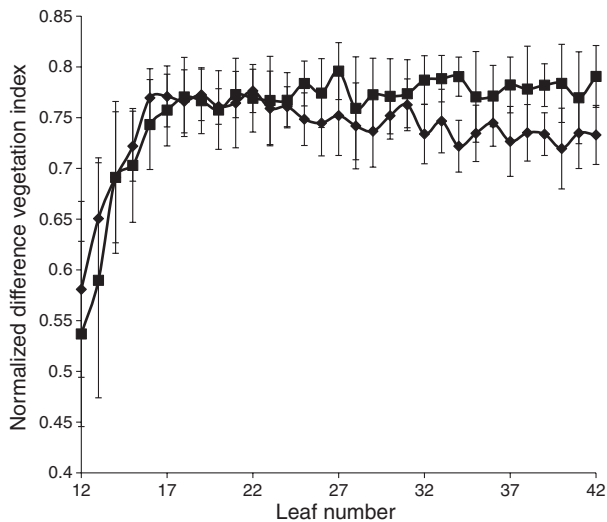


Figure 4. Normalized difference of vegetation index (NDVI) comparison between wild-type and fuzzy leaves. NDVI values for wild-type leaves (black diamonds) compared with fuzzy leaf NDVI values (black squares) show no significant difference in the light absorbance by young leaves of fuzzy and wild-type plants (leaves 12–32), but demonstrate a significantly higher absorbance of light by fuzzy leaves 33–42 as compared with wild-type ($P < 0.05$). Error \pm SD.

also had an increased photosynthetic capacity, elevated rate of transpiration and enhanced growth rate is surprising. The application of a tree with such phenotypes in forestry could be an interesting direction for future research.

The poplar gene *PtaMYB186* is responsible for the fuzzy phenotype

The misregulated gene that gave rise to the fuzzy phenotype was *PtaMYB186*, a close homologue of other R2R3 MYB transcription factors known to affect trichome morphogenesis. Three classes of MYB transcription factors (R1R2R3, R2R3 and R3) are grouped according to the presence of a highly conserved N-terminal DNA binding domain, whereas their C-terminal domains vary widely based on their role in transcriptional regulation (Kranz *et al.*, 1998; Jin and Martin, 1999; Meissner *et al.*, 1999). The R2R3s comprise the largest family of transcription factors in plants (Riechmann *et al.*, 2000), and have a number of members that control trichome morphogenesis (Oppenheimer *et al.*, 1991; Noda *et al.*, 1994; Kirik *et al.*, 2001; 2005). A recent annotation of the R2R3-MYB family in *Populus trichocarpa* identified 193 members, more than any other plant with a sequenced genome to date (Wilkins *et al.*, 2009a).

The *PtaMYB186* gene misregulated in the fuzzy mutant bears closest sequence homology with the Arabidopsis *AtMYB106*, a repressor of trichome outgrowth and branching (Jakoby *et al.*, 2008). As overexpression of *PtaMYB186* recapitulated the increased trichome phenotype of the fuzzy mutant, this would suggest that the *PtaMYB186* protein functions more like *AmMIXTA*, a close homologue of

AtMYB106, which has been shown to promote trichome differentiation (Glover *et al.*, 1998). A cotton homologue of *PtaMYB186* (*GhMYB25*) also promoted trichome initiation (Wu *et al.*, 2006). Similarly, enhanced MYB expression induced trichome differentiation in some species, but not in others (Payne *et al.*, 1999; Gruber *et al.*, 2006).

PtaMYB186 is a member of the *Populus* R2R3-MYB clade 15 (Wilkins *et al.*, 2009a). In addition to *PtrMYB186*, clade 15 includes four *Populus* R2R3-MYB genes (*PtrMYB039*, *PtrMYB083*, *PtrMYB089* and *PtrMYB138*), two *A. thaliana* genes (*AtMYB16* and *AtMYB106*), two *Vitis vinifera* genes (*Vv14g18963442* and *Vv17g7086341*) and one *Petunia hybrida* gene (*PhMYB1*) (Figure 7). Strikingly, the *PtaMYB186* subgroup is expanded in poplar relative to both *A. thaliana* and grapevine. The expansion of this subgroup in poplar suggests that there is either functional redundancy in poplar, or that the poplar family members have assumed diverse functions in poplar growth and development. The potential for redundancy emphasizes the value of activation tagging as a gene discovery approach, as the role of *PtaMYB186* might not have been uncovered in a loss-of-function approach, where other members in the clade might compensate for the absence of *PtaMYB186* activity.

Potential functional redundancy in the *PtaMYB186* subgroup during the development of emerging leaves is suggested by transcript abundance data from the PopGenExpress data set (Wilkins *et al.*, 2008), where the highest transcript abundance occurs in this organ relative to other tissues/conditions for both *PtrMYB186* paralogues (probe set: PtpAffx.85736.1.S1_at) and *PtrMYB083* (probe set: PtpAffx.28813.1.S1_at) (Figures S2 and S3; Table S5). Nevertheless, functional divergence is also suggested by the transcript abundance data, as *PtrMYB186* (probe set: PtpAffx.85736.1.S1_at) also shows a high level of transcript abundance in male catkins relative to other tissues (except young leaves), in contrast to *PtrMYB083* (probe set: PtpAffx.28813.1.S1_at), which does not show appreciable transcript accumulation in this tissue (Figures S2 and S3; Table S5). Notably, transcript accumulation at the PtpAffx.85736.1.S1_at probe set was significantly higher in the shoot apices of fuzzy than in the apices of wild-type plants ($\log_2FC = 0.95$, one-sided Student's *t*-test, $t = -5.8$, d.f. = 4, $P = 0.002$). *PtaMYB083* (PtpAffx.28813.1.S1_at), which is distinguishable from *PtaMYB186* using the Affymetrix GeneChip, was expressed at significantly lower levels in fuzzy than in wild-type plants ($\log_2FC = 1.19$, two-sided Student's *t*-test, $t = -2.9$, d.f. = 4, $P = 0.05$).

PtaMYB186 misexpression alters transcript abundance in shoot apices

Whole-transcriptome microarray analysis of genes with significant differences in transcript abundance in fuzzy mutants relative to wild-type plants revealed some genes in

Figure 5. The *fuzzy* mutant exhibits an increased growth rate.

(a) Mutant line to left of the image shown after 80 days of growth, as compared with the wild type on the right.
 (b) Growth rate of tree height of mutant (black squares) compared with wild-type poplar (black diamonds) from bud break to 1.59 m.
 (c) Growth rate of stem diameter of mutant (black squares) versus wild-type poplar (black diamonds) taken at 30 cm above ground, over a tree height range of 46 cm–1.59 m. Error \pm SD.

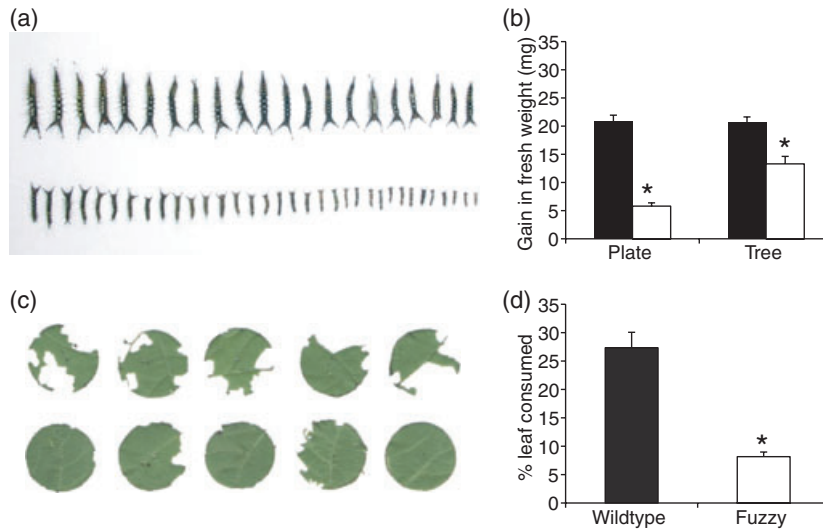
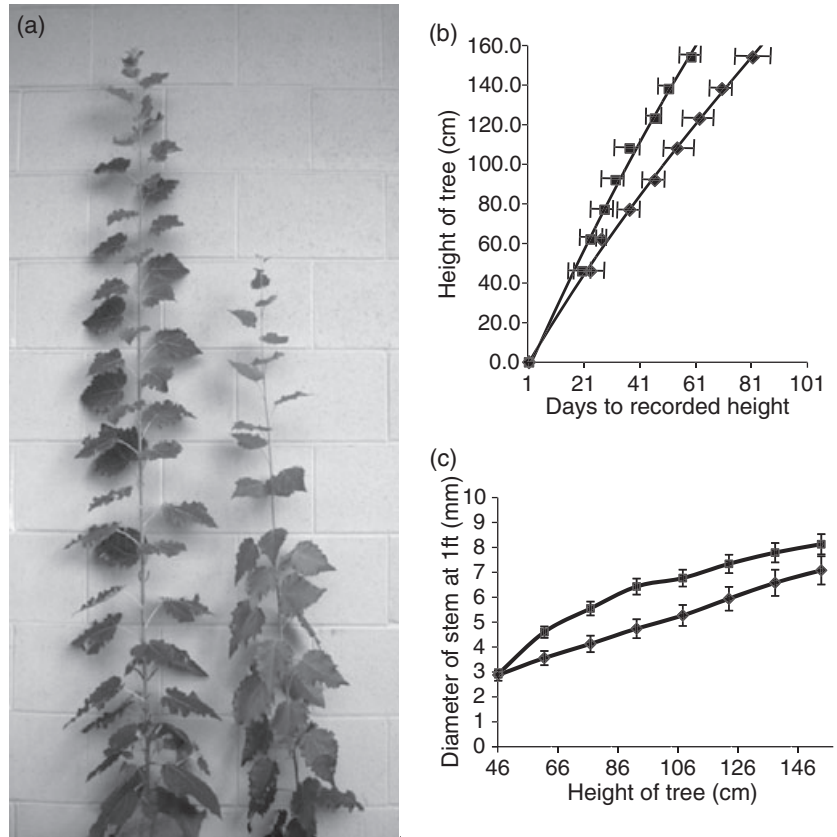


Figure 6. *fuzzy* exhibits increased resistance to tussock moth larvae feeding.

(a) Larvae reared from first instar for 14 days on either detached wild-type poplar leaves (top row) or *fuzzy* leaves (bottom row).
 (b) Gain in fresh weight of larvae reared from hatching for 14 days on detached leaf discs of wild-type leaves (black bar) versus *fuzzy* leaf discs (white bar) (plate trial), or raised from second-instar larvae for 14 days caged on wild-type or *fuzzy* trees (tree trial).
 (c) Representative examples of leaf disc damage 24 h after the initiation of feeding in choice assays between wild-type (top row) and *fuzzy* (bottom row) leaf discs.
 (d) Average percentage leaf area consumed for wild-type (black bar) and *fuzzy* (white bar) leaf discs in choice feeding assays. *Statistically significant difference from wild type ($P < 0.05$, Student's *t*-test). Error \pm SE.

which function correlates well with the *fuzzy* phenotype. For example, a suite of genes encoding heat-shock proteins were prominent amongst the genes with increased tran-

script abundance in the *fuzzy* mutant. Consistent with this, genes encoding heat-shock proteins have been previously implicated in trichome differentiation (Jakoby *et al.*, 2008;

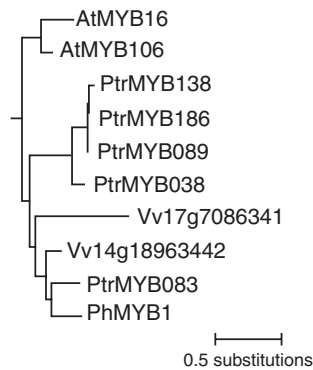


Figure 7. *fuzzy* (*PtrMYB186*) is part of *PtrR2R3-MYB* clade 15. Clade 15 includes closely related R2R3-MYB transcription factors from the genomes of *Arabidopsis thaliana* (*At*), *Populus trichocarpa* (*Ptr*), *Vitis vinifera* (*Vv*) and *Petunia × hybrida* (*Ph*). The phylogeny is modified from Wilkins *et al.* 2008. Scale bar = 0.5 substitutions per site.

Marks *et al.*, 2009). Similarly, several regulatory genes previously implicated in the control of *A. thaliana* trichome formation, including *KAKTUS* and *AtMYB4*, also had increased transcript abundance in the *fuzzy* mutant. In *A. thaliana*, these genes are typically associated with the control of trichome branching and morphology, as opposed to strictly trichome patterning (Perazza *et al.*, 1999; Downes *et al.*, 2003; El Refy *et al.*, 2004). As poplar trichomes are not branched, the increased transcript abundance of *KAKTUS* and *AtMYB4* in *fuzzy* mutants suggests that these genes may have been adopted for a different function in trichome development in poplar. Our findings therefore emphasize the need for functional characterization of genes implicated in trichome formation in several plant systems, rather than relying on simple homology searches.

Analysis of transcriptome remodelling in shoot apices of the *fuzzy* mutant relative to wild-type poplar shoot apices suggested that the transcript abundance of genes involved in the regulation and perception of hormones differed between the two genotypes. In *fuzzy*, the transcript abundance of genes associated with auxin flow and breakdown was lower than that in wild type, as was the transcript abundance for ethylene response genes and a gene encoding the ethylene receptor ETHYLENE RESPONSE SENSOR 1. Jakoby *et al.* (2008) found that ethylene-responsive genes were differentially regulated in trichomes relative to epidermal pavement cells, and others have found that ethylene affected trichome spacing (Kazama *et al.*, 2004) and trichome branching (Plett *et al.*, 2009). Taken together, it is possible that ethylene might be involved in regulating trichome development in poplar.

The *fuzzy* mutant exhibits increased photosynthesis and growth rate

Despite the increased carbon allocation resulting from a higher density of trichomes it was observed that the *fuzzy*

mutant also had increases in growth rate and in the rate of photosynthesis. Whole-transcriptome analysis to identify genes with altered transcript abundance in *fuzzy* relative to wild-type poplar indicated that genes typically associated with increased growth (i.e. cell wall synthesis, cell cycling genes) or photosynthesis were either unaffected or had a reduction in transcript abundance in the *fuzzy* mutant. Although there is still the possibility that one of the other genes with increased transcript abundance in *fuzzy* mutants would result in an increase in growth rate, an alternative hypothesis would be that trichome density may indirectly affect growth rate. Modelling and experimental techniques have demonstrated that trichomes can increase transpiration rate and CO₂ availability in plants by altering leaf boundary layers (Schreuder *et al.*, 2001; Benz and Martin, 2006). Thus, the increased availability of CO₂ in *fuzzy*, resulting from a decrease in the resistance to CO₂ (Table 1), could lead to a higher rate of photosynthesis, as has been previously demonstrated (Brewer and Smith, 1994; Drake *et al.*, 1997; Norby *et al.*, 1999; Nowak *et al.*, 2004; Ainsworth and Long, 2005; Liberloo *et al.*, 2006; Pereira de Souza *et al.*, 2008). As higher ambient CO₂ concentrations result in increases in poplar growth (Liberloo *et al.*, 2006), it is possible that the altered growth observed in *fuzzy* trees is a physiological product of the increased gas exchange caused by altered trichome canopy height and density. Although we are unable to prove this link, further testing will be required to determine if the altered trichome profile in *fuzzy* affects the boundary layer around the leaf surface and growth rate. It must be stressed, as well, that these growth trials were performed under ideal conditions, and it is unknown whether this increased growth rate would occur under field conditions and be maintained over several years of growth. This is currently being studied in a long-term field trial.

Increased trichome density reduces insect herbivory

Natural plant defences against insect herbivory typically involves the production of physical barriers to feeding, such as trichomes, or the production of bitter or toxic secondary metabolites. Whereas the decreased foraging by white-marked tussock moth larvae could result from the increase in trichome density on *fuzzy*, as has been found in other pubescent plants (Skaltsa *et al.*, 1994; Espigares and Peco, 1995), this preference could also result from an increased production of secondary metabolites (e.g. phenolic glucosides) or proteins (e.g. Kunitz protease inhibitors) that deter insect feeding, and/or delay development through reduced nutrition from leaves. Expression analysis of *fuzzy* shoot tips demonstrated that a number of genes associated with defence signalling, phenylpropanoid pathways and wax production were repressed in developing leaves (e.g. *LOX2*, *PRL1*; Table 2; Table S1). This may result, in part, from the overexpression of the Arabidopsis *MYB4* poplar homologue, which, other than being known as a homologue of

GL1, is a transcriptional repressor that modulates the expression of genes involved in phenylpropanoid biosynthesis (Jin *et al.*, 2000). Furthermore, genes associated with induced defences against feeding insects are not among the list of genes with increased transcript abundance in the *fuzzy* mutants. These data combined suggest that decreased feeding on *fuzzy* leaves by white-marked tussock moth larvae is probably the result of an increased density of trichomes acting as a physical deterrent, and not the result of an increase in the production of defensive metabolites or proteins.

CONCLUSION

Here, using an *a priori* screen of an activation-tagged poplar mutant population, we have identified a trichome regulatory gene (*PtaMYB186*) that, when activated, causes an increase in trichome density. These mutants also demonstrate increased transpiration, photosynthetic capacity, growth rate and insect resistance relative to wild-type plants. The closest homologue of this gene in *A. thaliana*, *AtMYB106*, has been characterized as a negative regulator of trichome branching. This work has emphasized the critical need for gene characterization within different model plant systems, as the pleiotropic impact of *PtaMYB186* on plant development would not have been predicted based on Arabidopsis research.

EXPERIMENTAL PROCEDURES

All plant material used for phenotypic characterization was grown under glasshouse conditions at Queen's University in Kingston, ON, Canada. The *fuzzy* mutant was generated as described in Harrison *et al.* (2007) in a *P. tremula* × *P. alba* clone 717-1B4 background, and all comparisons of *fuzzy* were made with this hybrid.

Identification and evaluation of the trichome phenotype

Leaf pubescence was compared among the activation-tagged population by an initial visual screen of trichome density, and was subsequently analyzed using cryo-scanning electron microscopy (SEM), as described in Harrison *et al.* (2007). Adaxial trichomes were counted from SEM images of fully expanded leaves. To determine the number of trichomes on the abaxial side of wild-type and *fuzzy* leaves, trichomes were removed from fully expanded leaves using adhesive tape, and impressions of the epidermis were taken using clear nail polish (Boeger *et al.*, 2004). Trichome stumps left within these impressions as well as stomates were counted using a Carl Zeiss Axioplan microscope (Carl Zeiss, <http://www.zeiss.com>), and were reported as number of trichomes or stomates per cm² ($n = 30$ leaves for wild type; $n = 28$ leaves for *fuzzy*). Because of the very high number of trichomes on poplar, and the difficulty that ensued for counting their density, we compared the density of stomates and epidermal cells between the wild type and *fuzzy* with values reported for other poplar species to gain confidence in our scoring. As the removal process damages the leaf surface, especially the contours of the epidermal cells, we only counted the replicates in which we could obtain accurate estimates of all cell types. As our data for stomatal density was well within the range reported for different clones of poplar (Ferris *et al.*, 2002; Dillen *et al.*, 2008; Woo, 2010), we are confident that this method of

density analysis is accurate. For each of the 35S::*PtaMYB186* lines at least three leaves of the same size were compared for trichome density to determine if trichome density was increased.

Identification of the gene responsible for the trichome phenotype

The single insertion site was determined by a modified TAIL PCR technique using the Genome Walker kit (Clontech, <http://www.clontech.com>) according to the manufacturer's instructions. The expression levels of three genes 10 kb upstream or downstream of the insertion site were tested in the shoot apex using the quantitative PCR ABI SYBR Green system, following the manufacturer's instructions (ABI, <http://www.appliedbiosystems.com>) using a Cepheid SmartCycler (Cepheid, <http://www.cepheid.com>). Primers used for the analyses were as follows: *PtaMYB186* forward, 5'-CTGCCTGCCAAGCTGGAC-3'; *PtaMYB186* reverse, 5'-GCAATG-GCTGACCACCTG-3'; putative E3 ligase, forward, 5'-GAGTGGTG-CTCAACAGGAG-3'; putative E3 ligase reverse, 5'-CCTCCCTTAC-TATTAGC-3'; putative ATP/GTP binding protein forward, 5'-GAG-TGGTGCTAACAGGG-3'; putative ATP/GTP binding protein reverse, 5'-CATTAGGACATTTAACCTC-3'; UBQ forward, 5'-TCCA-AGACAAGGAAGGCATCC; UBQ reverse, AGCACCAAGTGAAGG-TTGACTC-3'. All primers were designed to span an intron-exon barrier to avoid amplification of genomic DNA contamination, and were only found to amplify from a cDNA template. The *ubiquitin* gene used as a reference was tested under experimental conditions and was found to be stably expressed. All primers showed a linear response to template concentration. All experiments were performed on three biological replicates.

The full-length sequence of the *MYB186* gene was determined using a Clontech Marathon cDNA kit according to the manufacturer's instructions (Clontech). *PtaMYB186* was cloned into pCAM-BIA1305.1 and transformed into wild-type 717 poplar as described by Harrison *et al.* (2007). Six lines were recovered from the transformation of 400 explants, and the trichome initiation in young leaves was compared with wild-type lines to determine if the phenotype was recapitulated. Images of representative leaves for each 35S::*PtaMYB186* line were taken using a Carl Zeiss Stemi 2000-C dissecting microscope (Carl Zeiss).

RNA extraction and microarray hybridization

RNA extraction and transcript abundance analysis using the Affymetrix Poplar GeneChip were essentially as described previously (Wilkins *et al.*, 2009a,b). Briefly, plant material was ground to a fine powder under liquid nitrogen, and total RNA was extracted from each sample using the Trizol method (Invitrogen, <http://www.invitrogen.com>). RNA quality was determined electrophoretically. For each sample, 5 µg of total RNA was reverse transcribed (SuperScript II; Invitrogen), labelled and hybridized to the Poplar Genome Array according to the manufacturer's protocols (Affymetrix, <http://www.affymetrix.com>) at the Centre for the Analysis of Genome Evolution & Function at the University of Toronto. For each genotype, RNA was extracted from three replicate biological samples. Each sample included two shoot apices collected from the primary stem, and each sample was hybridized to an Affymetrix Poplar GeneChip.

Microarray analysis

GeneChip data analysis was performed using the BioConductor suite (Gentleman *et al.*, 2004) in R (R: A language and environment for statistical computing; <http://www.R-project.org>) (R Development Core Team, 2009) using the *AFFY* package (Gautier *et al.*, 2004). All

six microarrays were pre-processed together using GC-robust multi-array analysis (GCRMA) (Wu *et al.*, 2004). Expression data were filtered to remove probe sets reporting low transcript abundance and low variance across all arrays (Gentleman *et al.*, 2009) (minimum intensity of 100 on a minimum of two arrays, minimum interquartile range of 0.5 on the \log_2 -scale).

The pre-processed data were analysed using the TREAT (Student's *t*-test relative to a threshold (McCarthy and Smyth, 2009); function in LIMMA (Linear Models for Microarrays package (Smyth, 2005); in R (R Development Core Team, 2009). The linear model was parameterized by group means with a manually defined sum-to-zero contrast matrix to test directly for genes that had different levels of transcript accumulation between the two genotypes. TREAT tests whether the true differential expression for a given probe set is greater than a given threshold value or fold change. In this instance, differentially expressed genes were identified for which there was a minimum twofold difference for the transcript levels in wild type and *fuzzy* with a false discovery rate (Benjamini and Hochberg, 1995) of <0.05 . All CEL files have been uploaded to GEO. Data, description of experimental design, and experimental methods are available for download, under GEO accession number GSE21061.

Growth comparison on wild-type and fuzzy trees

Two separate growth trials were performed in early and mid-summer of 2007 in a glasshouse at Queen's University (44.23°N, 76.5°W) under natural day length. The average daily ambient air temperature for the first growth trial was $23.5 \pm 2.0^\circ\text{C}$, whereas for the second growth trial the average daily air temperature was $24.5 \pm 2.9^\circ\text{C}$. Ventilator fans were used to maintain an even temperature within the glasshouse at both the soil level and at the tree canopy level. Trees were watered daily and fertilized once a week with 20:20:20 (N : P : K) fertilizer (Plant Products Inc., <http://www.plantprod.com>). Stem cuttings of the same diameter and length of wild type ($n = 19$) and *fuzzy* ($n = 23$) were rooted by inserting a toothpick soaked in indole-3-butyric acid through the bottom of each cutting and grown in a 50:50 mix of sand and soil for 1 month. Once roots were established the cuttings were transferred to individual pots and shoots were reduced to one shoot per cutting. Once shoots reached 30 cm in height, the growth trial was initiated and the time for each tree to grow an additional 15 cm was recorded until trees reached a height of 150 cm. Tree trunk girth was measured 30 cm from the base of the growing stem, beginning when each tree was 45 cm in height until the end of the growth trial.

Photosynthesis and NDVI measurements

Rates of photosynthesis and respiration were analysed on the first fully expanded leaf of wild-type and *fuzzy* trees with the Qubit CO₂ analysis system (Qubit, <http://www.qubitsystems.com>) using an artificial light source and air containing 400 ppm CO₂, at a constant airflow of 0.5 L min^{-1} , and with the leaf temperature maintained at 24.8°C . Calculations were performed according to the manufacturer's instructions. The efficiency of water use was measured as the ratio of CO₂ exchange rate to the transpiration rate, as described by Kenzo *et al.* (2008). NDVI was performed using a handheld PLANTPEN (Qubit), according to the manufacturer's instructions.

Tussock moth developmental and feeding bioassays

Tussock moth egg masses were obtained from the Canadian Forestry Service and hatched in sterile Petri dishes. Newly emerged larvae were transferred to plastic rearing cups with General Purpose Lepidopteran diet media (BioServ, <http://www.bio-serv.com>), and left to feed until the larvae were at the desired larval instar. For

larvae developmental assays, six newly hatched larvae were placed in a Petri dish with four leaf discs of either *fuzzy* or wild type trees ($n = 8$ for wild-type; $n = 12$ for *fuzzy*). These leaf discs were replaced every 2–4 days to maintain a fresh supply of leaf matter and to maintain an excess of food for the larvae. After 14 days the larval instar and gain in fresh weight were recorded for each test. For the development of the larvae on trees, net cages were set up around 10 leaves of the same plastichron index on each *fuzzy* or wild type tree ($n = 6$ for wild type; $n = 7$ for *fuzzy*). Ten second-instar larvae were transferred into each cage and left to develop for 14 days. At the end of the trial larval instar and gain in fresh weight were analysed. For feeding preference tests, four-fourth-instar larvae that had been starved for 24 h were placed in a large Petri dish with four leaf discs of *fuzzy* trees and four leaf discs of wild-type trees, and left to feed for 24 h ($n = 5$). The remains of leaf discs were scanned and analysed for remaining leaf area using a custom-modified image processing program [GNU Image Manipulation Program (GIMP) v2.4.6; <http://www.gimp.org>].

Statistical analysis

A Student's *t*-test was performed to determine the significance of the results for comparison of *fuzzy* and wild-type physiological parameters, and in the analysis of the tussock moth larvae feeding challenges ($P < 0.05$). General statistical analyses of the microarray results were essentially as described previously (Wilkins *et al.*, 2009a,b), with the specifics pertinent to the analyses reported in the current work described in detail above.

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SUPPORTING INFORMATION

The following supplementary material is available for this article online:

Figure S1. Pearson correlation heat map of the *fuzzy* and wild-type shoot apex transcriptome.

Figure S2. Absolute transcript abundance data from Affymetrix GeneChip microarray analysis for *PtrMYB186* paralogues (probe set: PtpAffx.85736.1.S1_at) across the PopGenExpress developmental data set (Wilkins *et al.*, 2009a,b).

Figure S3. Absolute transcript abundance data from Affymetrix GeneChip microarray analysis for *PtrMYB083* (probe set: PtpAffx.28813.1.S1_at) across the PopGenExpress developmental data set (Wilkins *et al.*, 2009a,b).

Table S1. List of genes with reduced transcript abundance in the *fuzzy* mutant shoot apices in comparison with wild-type shoot apices.

Table S2. List of genes with increased transcript abundance in the *fuzzy* mutant shoot apices in comparison with wild-type shoot apices.

Table S3. GOslim annotation of 766 probe sets with higher levels of transcript accumulation in the wild type than in *fuzzy*.

Table S4. GOslim classification included 41 probe sets with higher levels of transcript accumulation in *fuzzy* than in wild type.

Table S5. Relative transcript abundance data from Affymetrix GeneChip microarray analysis for *PtrMYB186* subfamily members (probe set: PtpAffx.85736.1.S1_at) and *PtrMYB083* (probe set: PtpAffx.28813.1.S1_at) across the PopGenExpress developmental data set (Wilkins *et al.*, 2009a,b).

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