

# Aerobic methane formation in Grey poplar plants grown under sterile conditions

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#### Introduction



## Objections to the experimental design of Keppler et al. (2006), criticizing the use of static chambers and methane-free air: e.g.,

Kirschbaum *et al.* (2006), *Functional Plant Biology* **33**: 521–530 Dueck *et al.* (2007), *New Phytologist* **175**: 29–35

#### No observation of aerobic methane emission from plants: e.g.,

Dueck *et al.* (2007), *New Phytologist* **175**: 29–35 Beerling *et al.* (2008), *Global Change Biology* **14**: 1821–1826 Kirschbaum & Walcroft, *Biogeosciences* **5**: 1551–1558

#### Observation of aerobic methane emission from plants: e.g.,

Vigano *et al.* (2008), *Biogeosciences* **5**: 937–947 Wang *et al.* (2008), *Environmental Science* & *Technology* **42**: 62–68

#### Mechanisms of aerobic methane formation: e.g.,

Keppler *et al.* (2008), *New Phytologist* **178**: 808–814 McLeod *et al.* (2008), *New Phytologist* **180**: 124–132 Messenger *et al.* (2009), *Plant, Cell & Environment* **32**: 1–9

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**Open research questions** 

- Missing proof for the absence of methanogenic microorganisms potentially contributing to aerobic methane emission from plants
- Convincing evidence that aerobic methane originates in living plant material

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#### **Our experimental design**



- Plant species: Grey poplar (*Populus* x *canescens*, syn. *Populus tremula* x *P. alba*), derived from cell cultures under sterile conditions
- Plants on sterile medium in gas-tight flasks in CH<sub>4</sub>-free air
- Headspace was exchanged with synthetic air containing 20% of oxygen and 385 ppm <sup>13</sup>CO<sub>2</sub> (99 at% <sup>13</sup>C)
- Flasks were kept in glove box filled with pure N<sub>2</sub> for 33 days under a 16/8 h light/dark regime
- GC-IRMS analysis of methane in the headspace
- Molecular biological analysis of plant material and medium for the methyl coenzyme M reductase alpha subunit (*mcrA*) gene
- EA-IRMS of bulk plant material after end of the experiment

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#### **Plant material**





Wild type *Populus* × *canescens* (Aiton) Sm. (syn. *Populus tremula* × *P. alba*) lines, amplified by micro-propagation

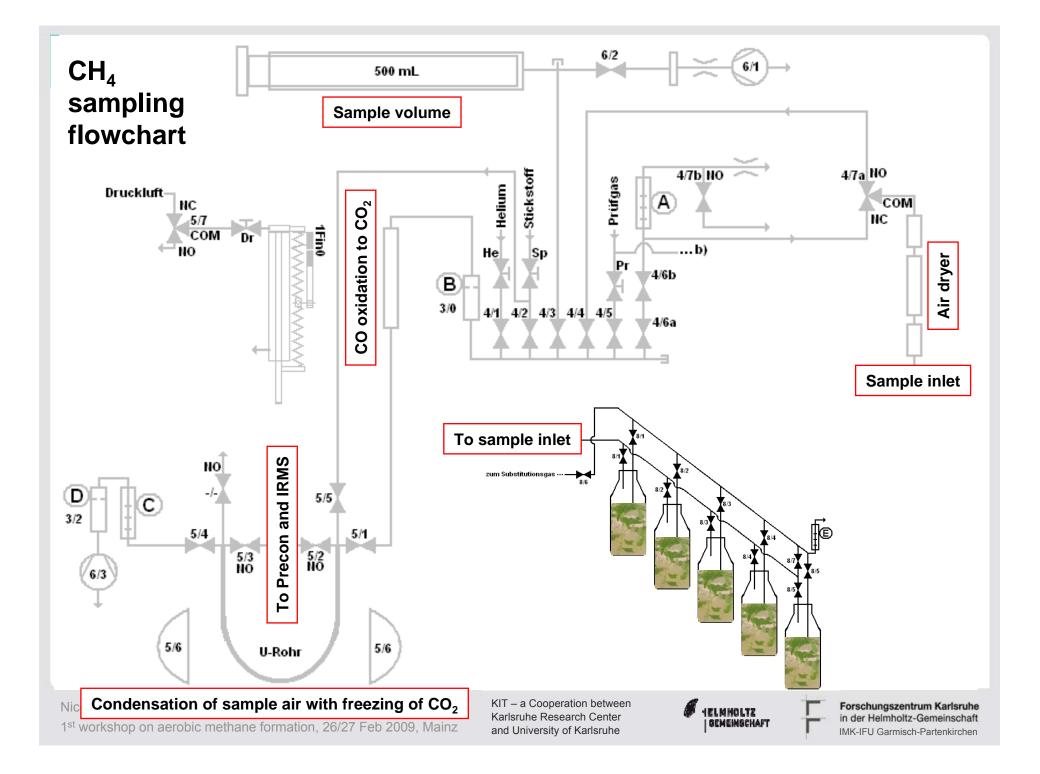
7-8 plantlets were transferred under sterile conditions to 1-I sterile glass flasks, containing sterilized quartz sand and MS medium

The flasks were sealed with screw caps and sterilized valves; the inlet ports were additionally equipped with sterile filters (0.22 µm pore size)

The poplar plants were grown under standard conditions of 27°C : 24°C (day : night) and a light period of 16 h with approx. 100  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> photosynthetic photon flux density (PPFD)

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#### mrcA primers used for PCR

Forward primer: GGATTCACACARTAYGCWACAGC

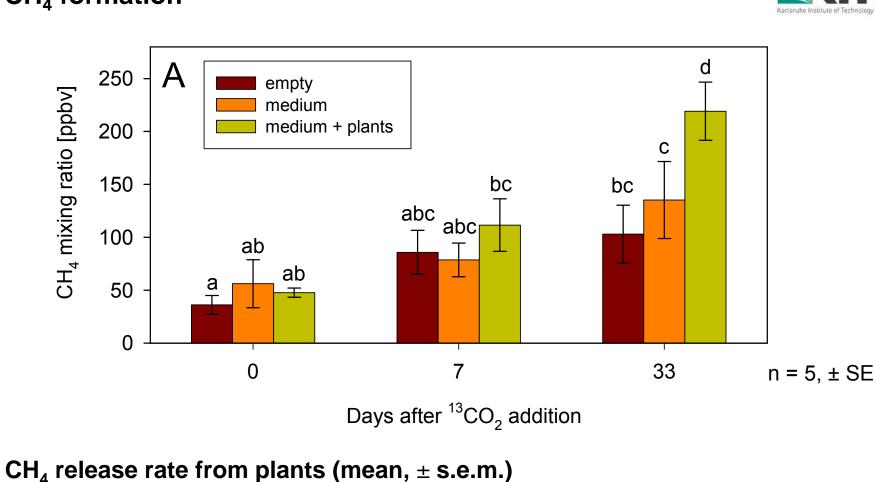
Databases: thousands of mcrA sequences but only "few" are full-length

Alignment: to
see conserved
regions and
design primers

sel=0	981							1135
AJ584650MeThAnosphAe	T AA TAACTTTA AA	ettatteeteeae <mark>ca</mark>	CACCATTATAC ACCAA TAT	TTA	TTCATACAT TCT	ТТ	T L ATTCACACAATAT CTACA C	CATACACA AT ARATATTA AT ACTTCATTTACTAT TARA ACT
NC 007681MeThAnosphA	T AA TAACTTTA AA	TTATT TO A CA	CACCATTATAC ACCAA TAT	TTA	TTCATACAT TCT	тет	TI ATTCACACRATAT CTACA C	CATACACA AT AAATATTA AT ACTTCATTTACTAT TAAA ACT
NC 000909MeThAnoCAld	ACA TAACATT A	TTATT T CA CA	CTACCTTTTAT ACCAA TTT	TTA	AA CTATAT TCT	A A	TI ATTTACACA TAT C TCA C	ACATACACA AT ACATCTTA AT ATTTT TTTATTAT AAT A
NC 009637MeThAnoCoCC	T AACAATCATTA AA	TA TA CAACT T	CT C TTATAC ACCAAATCT	CTC	T CATACAT TCT	ТТТ	T & ATTCACACAATAC CTACC C	TCATACACC AT ATATCTTA AT ACTTCTCATACTAC ATTA ATT
ABFP01000001MeThAnoC	T A CA TCATTA AA	TA TA CAACA T	CT CTTTATAC ACCAAATCT	CTC	T CATACAT TCT	ТТ	TA ATTCACACAATAT CTACC C	TCATACACC AC ATATCTTA AT ACTTCTCATACTAC ATTA ATT
NC 009135MeThAnoCoCC	T AACAATCATTA A	TT TA CTACTOT	CT CATTATAC ACCAAATCT	CTC	T CATACAT TCC	т т	T I ATTCACACAATAC CTACCIC	TCATACACC AT ATATTTTA AT ACTTCTCATACTACC ATTA ATT
NC 005791MeThAnoCoCC	T AACAATCATTA AA	TA TA CAACA T	CT CTTTATAT ACCA ATCT	CTC	T CATACAT TCT	ТТ	T & ATTCACTCRATAC CTACC C	TCATACACC AC ATATCTTA AT ACTTCTCATACTAC CACTC ACT
NC 009634MeThAnoCoCC	T A CA TCATTA AA	TA TA CA CTOT	CT CATTATAT ACCAAATAT	CTT	T CATACAT TCT	тс	T I OTTTCACACAATACCCTACA C	CATACACC AC ATATCTT AC ATTTCTCATACTAT CACTT ACT
ABHB01000001MeThAnoC	T A CAATCATTA A	TA TT CAACTOT	CT CTTTATAC ACCAAATCT	CTT	TTCATACAT TCT	ТСТ	TI ATTCACACATAT CTACA C	TCATACACA AT ACATCITA AT ACTICICATACIAC ATTA ACT
NC 009635MeThAnoCoCC	T A CAATCATTA A	TA TT CTACCE A	CTAT TTATAT ACCAAATCT	TTA	T CATACAT TCT	ТТ	TA ATTTACCCAATAT CAACA C	CATACACT AT ACATCITA AT ATTICT CTACTAT ATTA ACT
NC 000916MeThAnoTher	AA ATATCACTT A	TCATC CALLT CA	CA CACTCTAC ACCA TCT	CTC	CTCATACAT TCA	т	T TTTCACCCA TAC CAACA C	CCTACACC AC ACATCOTO AC ACTTO TATACTACI TATI A T
009990	AA ATATCACTT A	TCATC CALITICA	CA CACTCTAC ACCA TCT	CTC	CTCATACAT TCA	тот	T TTTCACCCA TAC CAACA C	CCTACACCE AC ACATCOTO AC ACTTO TATACTACE TATE A T
NC 009515MeThAnobrev	AC TTCAACCTTA AT	TA TA CTTTA T	CT CATTATAC ACCAAATTT	TTA	TTCTTACAT TCT	ТТ	TE ATTCACTCARTAT CTACT C	CATATACT AT AT TATTA AT ACTTCACTTACTAT TAAA ATT
AY386125MeThAnobACTe	TO T TA CTCTA AT	TT TA CATCTOT	CAAT TTATAC ACCAAATCT	CTC	ATCATACAT TCT	т т	T CATTCACACAATAT CTACCCC	CATACACC ACAACATTCTT AC ACTTCACCTACTAT TAAA AAT
DQ677519MeThAnobACTe	AA TAACACTC AT	TT TT CAACCET	CAAT TTATAC ACCA ATCT	CTC	ATCATACAT TCC	тет	T I CATTCACACA TACCCTACA C	CTTACACC ATAAT TTCTT AC ACTTCT TTACTACE TARA AAT
U10036	CACCETCTCACTURAC	TT T CAACCEET	CAAT CTCTAC ACCA ATCT	CTT	CTCATACAT TCA	ТТ	T I ATTCACCCA TAC CARCA C	CCTACACA ACAACATCCTC AC ACTTCACCTACTTC TAA A T
NC 008942hypoTheTiCA	AAAA CATCCCT AA	AAT TC CA CA CA	CCAT CTCTTC ACCA ATCT	CTC	ATCCTACAT TCC	COT	T COTTCACCCA TAC CAACT C	COTACACE ATAACATCOTT AT ATTTCACCTACE AAT FACT
NC 008942ribosomAlpr	AAAA CATCCCT AA	AAT TC CA CA A	CCAT CTCTTC ACCA ATCT	CTC	ATCCTATAT TCC	СПТ	T TTTCACCCA TRO CAACT C	CATACACH ATAACATCCTT AT ACTTT TATACA CH AATH ACT
NC 007796MeThAnospir		TTOTCOTCCACOT:	I TAT CTCTTC ACCA ATCT	CTC	TTCCTACAT TCT	т	T ATTTACCCA TAT CAACO C	CATACACC ACAACATCCTC AT A TTCACCTACTAT TAT
NC 009051MeThAnoCull	AAA CCTCCCTT A	TO TO COCCERT	ACCAT CTCTAC ACCA ATCT	CTC	CTCCTACAT TCC	CIC	T DESTRUCTION COLOR TAC CAACCEC	CCTACACC ACAACATCCTC AC A TTCACCTACTAC TATT ACT
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AY327049unCulTuredAr	CC CT CACACTC AA	TT TT C ACCEC	AC AT CTCTAC ACCA ATAT	CTT	ATCATACAT TCA	тет	T ATTCAC CA TAC CAACA C	CATACAC AAC AT TOT AT ACTTCAC TACTACE ATAC ACT
AY714839unCulTuredAr	CTT TACACACTC A	TO TT CTT C CC	AC AT CTCTAC ACCA ATAT	CTT	ATCATACAT TCA	тот	T : ATTCAC CA TAC C ACA C	CATACACCARCE AT TECT SEAT ACTTCAC TACTACES ATAC ACT
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AY260439MeThAnosArCi	ARA TO CTOTO AR	TO TRUTCASCO	I TAT CTCTAT ACCA ATCT	CTC	ATCCTACAT TCC	тет	T TTCACACA TAT CARCA C	CATACACCE AT ACATCOTT ACAACAACACCTACTACE AC TT ACT
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AY714816unCulTuredAr	AA AAT AATTA A 7	A CATC CA TT CT	CTATE TCTAC ACCA TT T	TTC	AAC TACAT TCT	A T	T & ATTCAC CA TAC CATCT C	ACATACACCERCARCATTCT FER SACTTCT CTACARCECTET ACA
AY714825unCulTuredAr	AA AAT AATTA A I	A CATC CA TT CT	CTAT TCTAC ACCA TT T	TTC	AAC TACAT TCT	AT	T & ATTCAC CA TAC CATCT C	ACATACACH ACAACATTCTHA HACTTCTHCTACAAHHECTHT-AHA
AY714837unCulTuredAr	AA AAT AATTA A I	A CATC CA TT CT	CTAT TCTAC ACCA TT T	TTC	AAC TACAT TCT	СПТ	T LEATTCAC CA TAC CATCT C	ACCTACACIE ATAATATCCTIER S ACTTCTICTACAASSECTET ALA
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BX649197unCulTuredAr	AA COT A TTA A	A CATC CA TT CT	CTAT TCTAT ACCA TT T	TTC	GAAC TACAT TOGE	A T	T LEATTCAC CA TAT C A T C	ACATACAC - ACAACATCCTA A - ACTTCT CTACAA - AT T A A
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AY714870unCulTuredAr	AA AAT AATTA A	A CATC CA TT CC	CAAT TCTAT ACCA TT T	TTC	TAAC TACAT TO G	TOT	T L ATTCAC CA TAT C A T C	ACATACAC SEACAACATCCT FEASEACTTCT CTACAA SEATET AFA

Reverse primer: TCATBGCRTAGTTHGGRTAGT

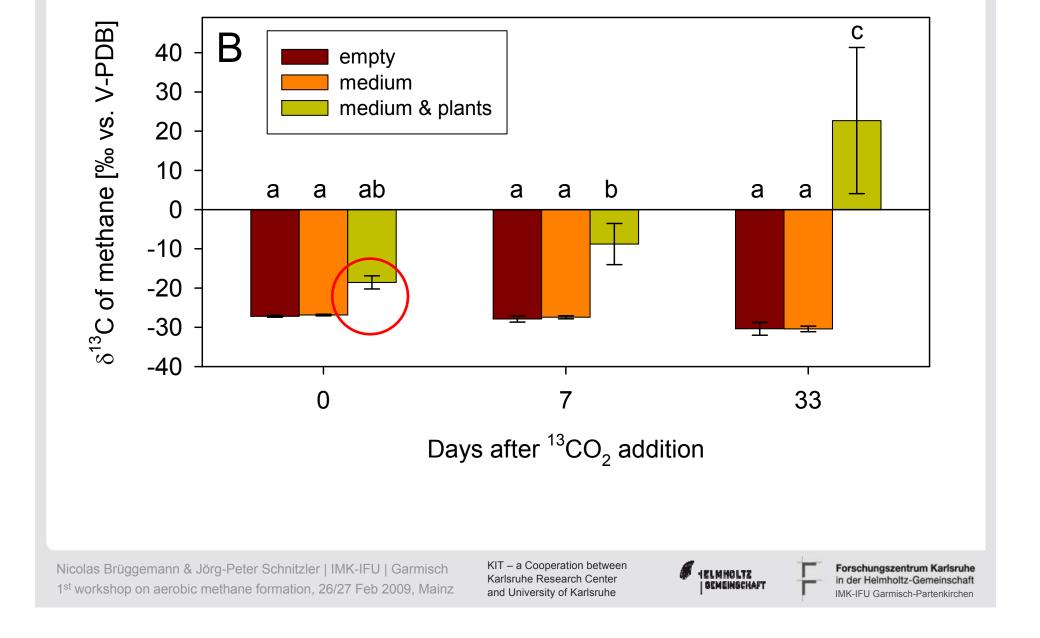
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U09990	CTCCCTCTCAAT	CATTA CTACTAT	-TCTCATACAC AACTCC T	ACCT ACTATCCARACTAC CCA	ТЕМ ІССТСССТ	CACCA CCA A TAC	CAN TATA CTCA CACCACAC CT CAASIS	C AT COTTOT TACAAACCCAC
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AY386125MeThAnobACTe	C TATTCTCAAT	TA A A AT AA	АСТАССАСТА ААСТТА А	A CA ACTACOCCARCTAT				
DQ677519MeThAnobACTe	C TATTT CAAT	AA COATAA	ATTACCTACA AATTAA A	CCCT ACTROCTRACTAC CCA	T6			
U10036	C TCTTCTCAAT	'AAGGEG <mark>C</mark> CACCAC	ACT CCACT A CTTC T	ACCT ACTATECHAACTAC CCA	TER CETEEET	CACCAGE TEATACE	CCI I TATATCACA I CACCACACI CTI CCCI TI	A AC COTTO T TTCAACCCAC
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A¥327049unCulTuredAr	C TAT CTC T	CCASSET SACEAS	AT CT CCTC AACTCC T	C CA ACTACCO AACTAC CAA	r a <mark>lo ta st</mark>	CACCALLIC A TAC	CA TATCACACA TCT CACACTCA CAACC	AC AT CATACT CT CAACCCAC
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AY714816unCulTuredAr		CCA A C AT A	AAT CCATTC A AT A A	ACTATCO AACTACOC A	TER ITETEECC	CATCA A T C TAC	CONTCTA TA COM A CACACT TO AATC	AC AT CAT TOT TOACCAT
AY714825unCulTuredAr		CCA A C AT A	CAAT CCATTC A AT A A	ICC.T. IACTATCO AACTACICIA	TEA ITETECCO	CATCA A T C TAC	СССТСТА ТА СССА САСАСТ Т С ААТС	ACCATOCATOGOTOCTOTCACCAT
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AY714852unCulTuredAr		CCA A C AT A	AAT CCATTC A AT A A	C TA ACTATCO RACTACIO A	THA COTCOCO	CATCA A T C TAC	C TCT TA C T CACACT T C AATC	AC AT CAT TT CT TCACCAT
BX649197unCulTuredAr		CCA A C AT A	GAAT CCATTC A AT A A	C TA HACTATCO AACTAC C A	TO A ICOTCOCO	CATCA A T C TAC	CC TCT TTAT A CACAC TT C AACA	AGE ACCCAT CONTACT TOACCAT
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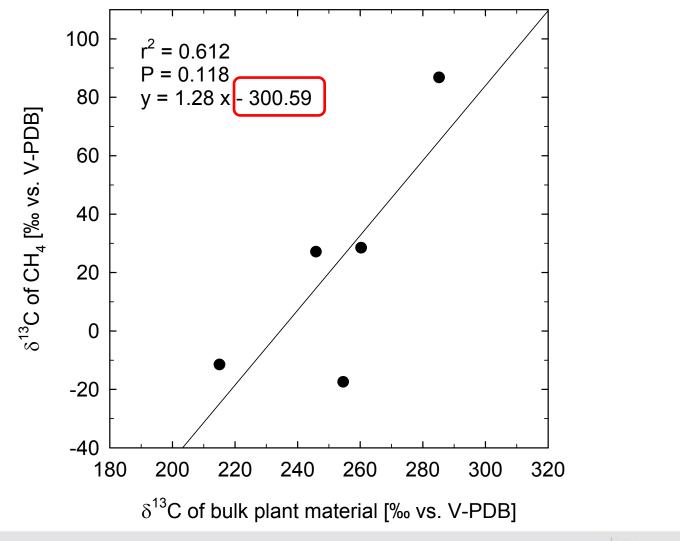


#### $\delta^{13}$ C of CH<sub>4</sub>

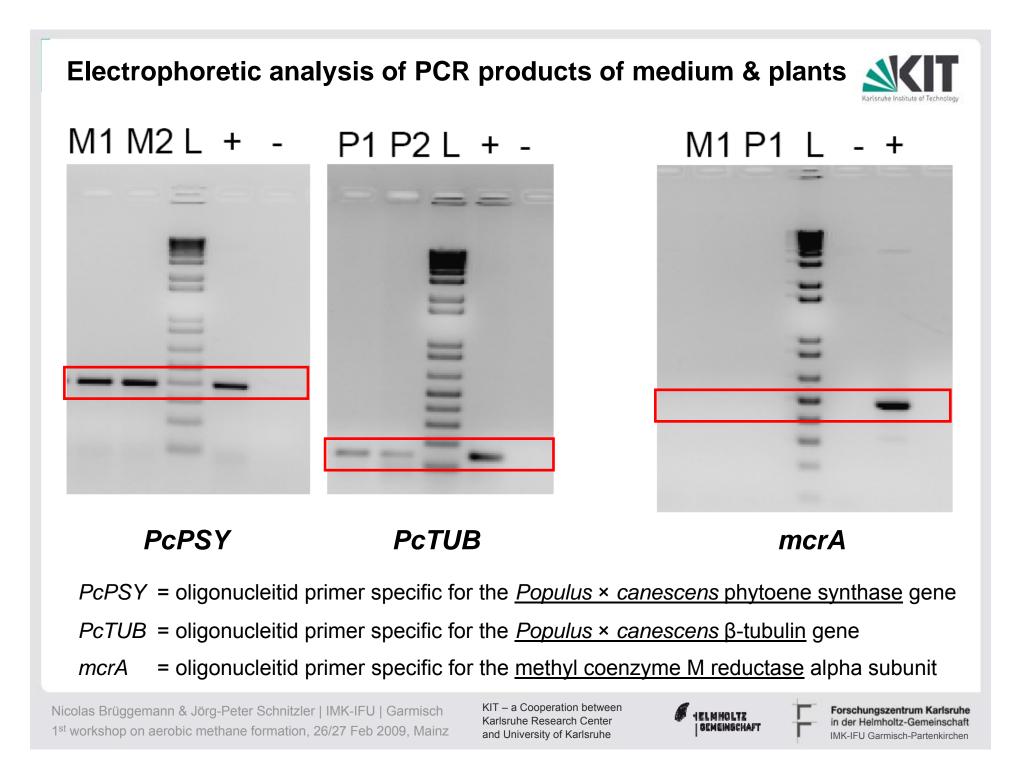


## Relationship between $\delta^{13}$ C-CH<sub>4</sub> and $\delta^{13}$ C of bulk plant material





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#### Range of aerobic CH<sub>4</sub> from living and detached plant material



ng CH <sub>4</sub> g <sup>-1</sup> DW h <sup>-1</sup>	References
ND	Kirschbaum & Walcroft, 2008; Nisbet et al., 2009, two species;
0.03	Vigano <i>et al.,</i> 2008, for a fully <sup>13</sup> C- labelled wheat leaf of Dueck et al., 2007, without UV light
0.16–0.7	Our work
0.5–13.5	Wang <i>et al.</i> , 2008, nine emitting species (35 non-emitting species)
-10-42	Dueck <i>et al.</i> , 2007, six species
(not significantly different from 0)	
Up to 32	Vigano <i>et al.,</i> 2008, for a fully <sup>13</sup> C- labelled wheat leaf of Dueck <i>et al.</i> , 2007, without UV light
32–49	Beerling <i>et al.</i> , 2008, two species
(not significantly different from 0)	
12–370	Keppler <i>et al.</i> , 2006, five species

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#### Summary



- We have observed release of <sup>13</sup>C-labelled CH<sub>4</sub> from poplar significantly different from zero under low (UV-free) light conditions after <sup>13</sup>CO<sub>2</sub> labelling
- The <sup>13</sup>C-label was detectable in CH<sub>4</sub> released from the plants already several minutes after start of <sup>13</sup>CO<sub>2</sub> labelling
- However, poplar methane emission rates are at the lower end of the reported CH<sub>4</sub> emission rates from living or detached plant material
- Our work is the first molecular biological proof for the absence of methanogenic microorganisms in plants emitting CH<sub>4</sub> under aerobic conditions

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### The "perfect" aerobic methane experiment?



### Goal:

Elucidation of CH4 mechanism(s) with simultaneous determination of realistic emission rates

- Experiments at ambient gas (CH<sub>4</sub>, O<sub>2</sub>, CO<sub>2</sub>) concentration levels
- Stable isotope labelling essential to differentiate between plant and atmospheric methane
- Analysis of plant-internal reactive oxygen species (ROS)
- Molecular biological verification of the absence of methanogenes
- Application of defined stress situations initiating ROS formation
- ...(open for discussion)

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