Induction of Resistance to *Verticillium dahliae* in *Arabidopsis thaliana* by the Biocontrol Agent K-165 and Pathogenesis-Related Proteins Gene Expression

Sotirios E. Tjamos,¹ Emmanouil Flemetakis,² Epaminondas J. Paplomatas,¹ and Panagiotis Katinakis²

Agricultural University of Athens, ¹Laboratory of Plant Pathology and ²Laboratory of Molecular Biology, 11855 Athens, Greece

Submitted 28 September 2004. Accepted 13 January 2005.

The biocontrol bacterium *Paenibacillus alvei* K165 has the ability to protect *Arabidopsis thaliana* against *Verticillium dahliae*. A direct antagonistic action of strain K165 against *V. dahliae* was ruled out, making it likely that K165-mediated protection results from induced systemic resistance (ISR) in the host. K165-mediated protection was tested in various *Arabidopsis* mutants and transgenic plants impaired in defense signaling pathways, including NahG (transgenic line degrading salicylic acid [SA]), *etr1-1* (insensitive to ethylene), *jar1-1* (insensitive to jasmonate), *npr1-1* (nonexpressing NPR1 protein), *pad3-1* (phytoalexin deficient), *pad4-1* (phytoalexin deficient), *eds5/sid1* (enhanced disease susceptibility), and *sid2* (SA-induction deficient). ISR was blocked in *Arabidopsis* mutants *npr1-1*, *eds5/sid1*, and *sid2*, indicating that components of the pathway from isochlorogenic acid and a functional NPR1 play a crucial role in the K165-mediated ISR. Furthermore, the concomitant activation and increased transient accumulation of the *PR-1*, *PR-2*, and *PR-5* genes were observed in the treatment in which both the inducing bacterial strain and the challenging pathogen were present in the rhizosphere of the *A. thaliana* plants.

Additional keywords: vascular wilt, plant-growth-promoting rhizobacterium.

Verticillium dahliae Kleb. is a destructive vascular wilt soil fungus widely distributed all over the world, causing great damage to many crops. The formation of resting structures known as microsclerotia is a critical factor in the survival, dissemination, and epidemiology of the disease. The prospects to control *Verticillium* wilt are to develop resistant cultivars or root stocks, new phloem-mobile fungicides, biological control agents, or compounds to induce resistance of the host plant (Tjamos 1989).

Several rhizosphere or endophytic bacteria have been reported as biocontrol agents against *V. dahliae* (Berg et al. 2001). The mechanisms of action of biocontrol agents against *V. dahliae* involve mainly antibiosis, parasitism, competition, and secretion of enzymes such as glucose oxidase, chitinase, and glucanase and induction of disease resistance in the host (Tjamos 2000).

In a previous study, Tjamos and associates (2004) reported the isolation of a plant-growth-promoting rhizobacterium (PGPR) strain, identified as *Paenibacillus alvei* and designated K165, with biocontrol activity against *V. dahliae* in glasshouse and field experiments. Root colonization by PGPR can result in induced resistance in many plants (Weller 1988). Induced systemic resistance (ISR) against the leaf pathogen *P. syringae* pv. *tomato* can be induced in *Arabidopsis thaliana* by the PGPR strain *Pseudomonas fluorescens* WCS 417r (Pieterse et al. 1998). These authors showed that ISR induced by WCS 417r was blocked in the *A. thaliana* jasmonate response mutant *jar1*, the ethylene response mutant *etr1*, and the nonexpressor of PR genes mutant *npr1*, indicating that components of the jasmonate and ethylene response as well as NPR1 have a crucial role in the ISR signaling pathway. Based on these results, the authors concluded that ISR mediated by PGPR is independent of SA activation of pathogenesis-related (PR) genes. These results only partially overlap with those of Iavicoli and associates (2003) in which the biocontrol rhizobacterium *Pseudomonas fluorescens* CHA0 was used to protect *Arabidopsis* against *Peronospora parasitica*. In this case, the protection depended on a full sensitivity to jasmonic acid on functional ETR1 and NPR1 proteins (Iavicoli et al. 2003).

The onset of systemic acquired resistance is accompanied by a local and systemic increase in the endogenous levels of SA and the subsequent upregulation of a large set of genes including the *PR-1*, *PR-2*, and *PR-5* genes (Van Loon and Van Strien 1999; Ward et al. 1991). Each group of PR proteins has a basic subclass found in the plant cell vacuole and an acidic subclass usually found in the extracellular space that might be part of the first line of defense to pathogens. Each group has members with antifungal activity and cognates of most groups have been found in a diversity of other organisms (Selitrennikoff 2001).

The main objectives of this work were to i) determine if induced resistance is the mode of action of the PGPR strain K165 against *V. dahliae*, by using the model plant *A. thaliana* and a novel bioassay, ii) elucidate the signaling pathway controlling the K165-mediated induced resistance, and iii) monitor the gene expression level of the acidic isoforms of the genes encoding PR-1, PR-2, and PR-5.

RESULTS

K165-mediated protection against *V. dahliae*.

The soilborne fungus *V. dahliae* is the causal agent of vascular wilt in a wide range of host plants, including the model plant *A. thaliana*. To study whether the induction of plant resistance is the mechanism for biological control of *V. dahliae*

Corresponding author: S. E. Tjamos; E-mail: stjamos@yahoo.com

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by the PGPR strain K165, a system ensuring spatial separation of the inducing agent and the challenging root pathogen, similar to that described by Leeman and associates (1995), was used.

As shown in Figure 1, the PGPR strain K165 conferred resistance to *A. thaliana* wild-type (wt) plants against *V. dahliae*. *Verticillium* wilt symptoms were visible 8 days postinoculation (dpi) (Fig. 2). In control plants, the percentage of infected leaves increased to 74% at 28 dpi, compared with 36% in bacterized plants (Fig. 1). Thus, colonization of the rhizosphere by K165 resulted in statistically significant reduction of disease severity.

To study whether the bacterial strain K165 and *V. dahliae* remained spatially separated during the experiment, the presence of K165 in the upper part of the roots (pathogen inoculation zone) and in the interior of the stem was evaluated at the end of each experiment. Rifampicin-resistant K165 bacteria were recovered from neither the upper part of the roots nor the stem, indicating that the biocontrol bacteria could not interact directly with the pathogen. This observation was made for the experiments presented in Figure 2 as well as those in Figures 3 to 6.

Throughout all experiments, mock-inoculated control plants remained healthy, and no apparent differences in the growth and development between controls and K165-treated plants were observed.

The protective effect of the treatment with K165 bacteria could result either from the induction of resistance in the plant or from an antibiotic effect of some factors produced by the bacteria and transported through the plant. A direct antibiotic activity would not, in principle, involve defense signaling pathways of the host. We have therefore examined the molecular requirements in the host for the expression of K165-mediated protection, with a special emphasis on the known disease resistance signaling pathways.

Molecular requirements

for the K165-mediated protection against *V. dahliae*.

The signaling pathway of K165-mediated induced resistance was investigated using the *A. thaliana* ethylene insensitive (*etr1-1*), the jasmonate insensitive (*jar1-1*), the nonexpresser of PR genes (*npr1-1*), the SA induction deficient (*sid2-1*), the enhanced disease susceptibility 5/SA induction deficient 1 (*eds5/sid1*), the phytoalexin deficient 3 (*pad3-1*), and the phy-

toalexin deficient 4 (*pad4-1*) mutants and the transgenic NahG plant impaired in SA accumulation.

Figure 3 shows that the ethylene-insensitive plants were protected against *V. dahliae* after K165 treatment. *Verticillium* wilt symptoms were similar to those observed in *A. thaliana* wt plants at 8 dpi. The maximum difference in the diseased leaves between the two treatments was observed at 22 dpi, (36%, in the controls versus 10% in the bacterized plants). The final percentage of diseased leaves 28 dpi was 39% in controls and 11% in the K165-treated plants. The low percentage of diseased leaves in both control and bacterized plants is in agreement with the observations of Veronese and associates (2003) showing that ethylene insensitivity in *A. thaliana* plants leads to reduced chlorosis after *V. dahliae* infection.

The jasmonate insensitive (*jar1-1*) *A. thaliana* mutant plants could be protected by strain K165 (Fig. 4). Among all the examined plants in this study, untreated *jar1-1* mutants showed the highest percentage of diseased leaves (representing 21%) at 8 dpi. Thus, jasmonate insensitivity appears to enhance a rapid increase in the expression of the disease. The percentage of diseased leaves in *jar1-1* plants further increased somewhat steadily to reach 55% at 28 dpi in control and 29% in bacterized plants. This is lower than the maximum percentage of diseased leaves in wt plants.

The phytoalexin deficient *pad3-1* and *pad4-1* mutants were protected by root treatments with strain K165. In *pad3-1* plants, the bacterial strain reduced significantly the percentage of diseased leaves as compared with the controls starting at 14 dpi and at 12 dpi in *pad4-1*. The maximum difference in the percentage of diseased leaves between the treatments was observed at 22 dpi in *pad3-1* and at 20 dpi in *pad4-1* plants. At 28 dpi, the percentage of diseased leaves was quite similar between *pad3-1* and *pad4-1*. In the control treatment, the percentage of diseased leaves was 66% in *pad3-1* and 64% in *pad4-1* plants. In K165-treated plants, the percentage of diseased leaves was 25% in *pad3-1* and *pad4-1* plants. Thus, camalexin is unlikely to be involved in protection to *V. dahliae* mediated by strain K165.

Treatment of the SA-nonaccumulating NahG plants with strain K165 caused a statistically significant reduction of symptoms compared with the control treatment. The largest difference was observed at 20 dpi with 39% diseased plants in untreated controls and 8% in K165-treated plants. Thus, protection appears to be independent of the accumulation of SA. However, neither *eds5/sid1* nor *sid2-1* mutants developed pro-

Fig. 1. Protection against *Verticillium dahliae* induced by *Paenibacillus alvei* K165 in *Arabidopsis* Col-0 plants, expressed as percentage of diseased leaves. Vertical bars indicate standard errors ($P > 0.05$).

Fig. 2. *Verticillium dahliae*-infected *Arabidopsis thaliana* plants with wilted and chlorotic leaves. Arrows point to leaves showing *Verticillium* wilt symptoms.

tection upon root colonization by strain K165. The percentage of diseased leaves in control and K165-treated plants reached 92% in *sid2-1* and 72% in *eds5/sid1* mutant plants at 28 dpi.

In addition, K165-treated *npr1-1*, like the *eds5/sid1* and *sid2-1* mutants, failed to be protected against *V. dahliae*. At 28 dpi, the disease severity in both treated and controls was close to 52%. Therefore, functional EDS5/SID1, SID2, and NPR1 are required for the development of K165-mediated protection.

Population dynamics of K165.

To investigate whether the lack of protection in the *npr1-1*, *eds5/sid1*, and *sid2-1* mutants was caused by insufficient colonization of the rhizosphere by strain K165, we determined the titer of rifampicin-resistant K165 at 8, 16, and 24 days after the application of the bacteria. Figure 5 shows that similar amounts of K165 bacteria were recovered from the induction treatment zone of Col-0, *etr1-1*, *jar1-1*, *npr1-1*, *pad3-1*, *pad4-1*, *sid2*, and *sid1/eds5*, representing a mean population density of about 10^6 CFU per gram of rhizosphere. Therefore, the loss of the capacity to express K165-mediated protection in *npr1-1*, *sid1/eds5*, and *sid2* is not caused by changes in bacterial root colonization but must be the result of alterations in the mutants.

Expression of *PR-1*, *PR-2*, and *PR-5* genes.

Gene expression of the *PR-1*, *PR-2*, and *PR-5* genes was investigated by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) in *A. thaliana* Col-0 plants, the roots of which were treated with the K165 strain and *V. dahliae*.

The mRNA level of the *PR-2* gene was induced at 6 dpi in the K165-*V. dahliae* treatment, compared with the control. Similarly, but to a lesser extent, the *PR-5* gene was also induced more after both K165 and *V. dahliae* treatments. These changes might reflect conditioning (Conrath et al. 2002) of *PR-2* and *PR-5* expression by the bacterial pretreatment, since K165 alone had, respectively, no or less effect. Interestingly, a relatively high accumulation of *PR-1* and *PR-5* transcripts in the control plants was observed at 6 dpi. A similar *PR-1* and *PR-5* gene upregulation in control untreated plants has been reported by Gaspar and associates (2004). The most likely explanation for the observed *PR-1* and *PR-5* transcript accumulation is that wounding or other stresses caused during the transplantation process may have triggered the expression of the corresponding genes. At 14 dpi, the expression of *PR-1*, *PR-2*, and *PR-5* mRNA was increased in the *V. dahliae* and the *V. dahliae*-

Fig. 3. Protection against *Verticillium dahliae* induced by *Paenibacillus alvei* K165 in *Arabidopsis etr1*, *jar1*, *pad3*, and *pad4* mutants, expressed as percentage of diseased leaves. Vertical bars indicate standard errors ($P > 0.05$).

K165 treatments, compared with the controls. Only the expression of *PR-2* mRNA showed a conditioning effect by the K165 treatment. The same conditioning was also observed for the expression of *PR-1*, *PR-2*, and *PR-5* at 20 dpi. Taken together, these data indicate a response of plants to pretreatment with K165.

DISCUSSION

The biocontrol activity of the *Paenibacillus alvei* K165 strain against *Verticillium* wilt of solanaceous hosts was previously demonstrated in glasshouse and field experiments (Tjamos et al. 2004). In the present study, we described the activity of strain K165 against the vascular wilt pathogen *V. dahliae* in the genetic system *A. thaliana* and characterized the molecular determinants in the host for the protection by strain K165. Strain K165 significantly reduced the development of vascular wilt symptoms in *A. thaliana* (Fig. 1). Because the inducing bacterial strain and the challenging pathogen remained spatially separated throughout the experiment, an antagonism by direct interaction can be ruled out. The loss of protection in the *eds5/sid1*, *sid2*, and *npr1* mutants strongly argues in favor

of an effect mediated through the plant. Therefore, we propose that strain K165 protects *Arabidopsis* against *Verticillium* wilt by inducing ISR.

Interestingly, ISR triggered by strain K165 differs from ISR triggered in *Arabidopsis* by *Pseudomonas fluorescens* WCS417r or CHA0 (Iavicoli et al. 2003; Pieterse et al. 1998). Whereas ISR triggered by strain K165 is SA-dependent, the resistance induced by the *P. fluorescens* strains was fully independent of SA. This demonstrates that PGPR-mediated ISR is likely to involve a variety of signaling routes, depending on the biocontrol agent and the target pathogen.

ISR induced by strain K165 against *V. dahliae* was blocked in *Arabidopsis* mutants *sid1/eds5*, *sid2*, and *npr1-1* (Figs. 3 and 4), indicating that components of the pathway from isochorismate, most likely SA, as well as a functional NPR1 play a crucial role in K165-induced ISR. This result appears somewhat difficult to reconcile with the full activity of K165-mediated ISR in NahG plants. Besides a low content of SA, NahG plants were shown previously to exhibit many pleiotropic changes in relation to defense signaling (Heck et al. 2003; van Wees and Glazebrook 2003). The production of hydrogen peroxide by the NAHG-mediated formation of catechol from SA

Fig. 4. Protection against *Verticillium dahliae* induced by *Paenibacillus alvei* K165 in *Arabidopsis* NahG transgenic plants and *npr1*, *sid2*, and *eds5/sid1* mutants, expressed as percentage of diseased leaves. Vertical bars indicate standard errors ($P > 0.05$).

(Schweigert et al. 2001) could at least partially account for this discrepancy, as H_2O_2 is known as a positive regulator of disease resistance in plants (Levine et al. 1994). Moreover, Wu and associates (1997) found that activation of multiple defense mechanisms by elevated levels of H_2O_2 in transgenic potato plants is likely involved in *V. dahliae* resistance. It remains unclear, if this could explain the behavior of NahG plants with respect to K165-mediated ISR, and further work is required to settle this point.

It was demonstrated that K165-mediated ISR is also dependent on NPR1. NPR1 (also referred as NIM1) is required downstream of SA to activate the expression of PR genes (Cao et al. 1997; Ryals et al. 1997). SA accumulation causes the translocation of NPR1 into the nucleus by changing the redox

status of the cell. In the nucleus, NPR1 interacts with members of the TGA family and enhances the binding of these factors to SA-response elements in the promoters of PR genes. It is likely that NPR1 controls the expression of the genes encoding PR-1, PR-2, and PR-5 (Eckardt 2003; Pieterse and Van Loon 2004). Since the protection induced by K165 is already expressed before 8 dpi, PR-2 and, to a lesser extent, PR-5 might be possible determinants for the resistance, since K165-treated plants show a stronger induction after inoculation with *V. dahliae*. The β -1,3-glucanase activity of PR-2 might weaken the fungal cell wall and prevent hyphal colonization. On the other hand, reaction products of their enzymatic activity may act as elicitors of host defense responses (Menu-Bouaouiche et al. 2003). The PR-5 family includes proteins that are homologous

Fig. 5. Titer of *Paenibacillus alvei* K165 in the rhizosphere of *Arabidopsis* NahG transgenic plants and *etr1*, *jar1*, *pad3*, *pad4*, *npr1*, *sid2*, and *eds5/sid1* mutants. Vertical bars indicate standard errors ($P > 0.05$).

Fig. 6. Expression of *PR-1*, *PR-2*, and *PR-5* in *Arabidopsis* Col-0 plants in response to infection with *Verticillium dahliae* or treatment with *Paenibacillus alvei* K165. RNA was isolated from roots of *A. thaliana* plants 6, 14, and 20 days postinoculation (dpi) and was analyzed by semiquantitative reverse transcription-polymerase chain reaction.

to the sweet-tasting protein thaumatin with demonstrated *in vitro* antifungal activity against *V. dahliae* (Abad et al. 1996; Pressey 1997). While an effect of PR-1 is unlikely to affect fungal growth, given its late induction after K165 treatment, it nevertheless provides a good indication for the induction of potential defenses by the host.

In conclusion, this study indicates that K165-mediated ISR in *A. thaliana* against *V. dahliae* is controlled by an NPR1-dependent SA signaling pathway. It is the first case of ISR demonstrated against *V. dahliae* in *Arabidopsis*. This provides a novel experimental access to study resistance mechanisms against an important disease that has remained inaccessible for molecular investigations so far.

MATERIALS AND METHODS

Fungal culture.

V. dahliae isolated from *Raphanus sativus* was provided by E. Ligoxygakis (National Agricultural Research Institute, Crete, Greece). The fungus was maintained on potato dextrose agar at 4°C. For the induced resistance bioassay, a suspension of 5×10^6 conidia per milliliter of distilled sterile water was prepared from a culture grown for 5 days at 22°C in sucrose sodium nitrate liquid medium.

Bacterial culture.

A rifampicin-resistant mutant of the biocontrol strain K165 with biocontrol activity against *V. dahliae* in glasshouse and field experiments was used in this study (Tjamos et al. 2004). K165 was applied in the induced resistance bioassay as a dust formulation. The antagonist was grown in liquid culture of nutrient broth and glycerol (Leliot and Stead 1987) in an orbital incubator at 180 rpm at 30°C for 18 h. Bacterial suspension was centrifuged at 8,000 rpm for 10 min, was resuspended in 50 mM phosphate buffer, pH 7.02, and a bacterial formulation of 10% gum xanthan-talc was prepared according to Kloepper and Schroth (1981). The concentration of the biocontrol agent in the dust formulation was 10^8 CFU per g of talc.

Origin of seeds.

Seeds of *A. thaliana* ecotype Columbia (Col-0) and the transgenic line NahG (Delaney et al. 1994) were provided by Novartis Crop Protection, Inc., (Greensboro, NC, U.S.A.); Col-0 accession mutants *etr1-1* (Bleecker et al. 1988), *jar1-1* (Staswick et al. 1992), *npr1-1* (Cao et al. 1997), *pad3-1* (Glazebrook and Ausubel 1994), and *pad4-1* (Glazebrook et al. 1996) were obtained from the Nottingham Arabidopsis Stock Center (Nottingham, U.K.); *sid2* and *eds5/sid1* (Nawrath and Métraux 1999) were provided by J. P. Métraux (University of Fribourg, Switzerland). All seeds were stored at 4°C.

Induced resistance bioassay.

A. thaliana seeds were sown in $9 \times 9 \times 10$ -cm pots containing sterile soil and were maintained at $21 \pm 3^\circ\text{C}$, 70% relative humidity, and a 12-hr light and dark cycle. After two weeks, seedlings were transferred to a plexiglas system containing two spatially separated compartments similar to the bioassay element described by Leeman and associates (1995), allowing an induction treatment and a challenge inoculation of the same root system. The plexiglas bioassay element consisted of two separate compartments ($60 \times 4 \times 6$ cm) containing sterile soil. The compartments were placed opposite each other and, at the touching sides, were vertically incised. The *A. thaliana* seedlings were placed horizontally on the two compartments through the vertical incision, the upper part of the roots on one compartment and the lower part, on the other. The lower part of the roots was covered

with either 1 g of K165 dust formulation, 10^8 CFU per gram of talc, or 10% gum xanthan-talc. Seven days after the induction treatment, plants were inoculated with *V. dahliae* by root drenching 1 ml of 5×10^6 conidia per milliliter to the upper part of the roots. Control plants were mock-inoculated with 1 ml of sterile distilled H_2O .

Disease severity, expressed as percentage of diseased leaves over the total number of leaves per plant, was periodically recorded for 28 days after inoculation. The experiment was repeated three times with 15 replicates per experiment.

To study whether the bacterial strain is remaining spatially separated for the duration of the bioassays on the lower, bacterized part of the roots, colonization of the upper part of the roots (pathogen inoculation) and of the stem interior was checked at the end of each experiment. The upper part of the roots was ground in 50 mM phosphate buffer, pH 7.02, with an autoclaved mortar and pestle, and the suspension was plated onto nutrient broth agar glycerol (NAG) medium supplemented with cyclohexamide (100 g/ml) and rifampicin (100 g/ml). The same procedure was followed for the stem samples.

Population dynamics of K165.

Colonization of the rhizosphere of wild-type, transgenic, and mutant plants by rifampicin-resistant K165 bacteria was determined at 8, 16, and 24 days after the application of the bacterial strain. To estimate rhizosphere populations, 0.5 g of rhizosphere soil (soil particles in close contact with roots about 1 to 5 mm thick) was collected and shaken for 45 min in 50 mM phosphate buffer, pH 7.02, containing 0.02% Tween 20, and the suspension was plated onto NAG medium supplemented with cyclohexamide (100 g/ml) and rifampicin (100 g/ml). After a 48-h incubation at 30°C, the number of bacterial CFU per gram of rhizosphere soil was determined. The experiment was repeated three times with 15 replicates per experiment.

Determination of transcript levels using RT-PCR assay.

Root tissue from the pathogen-inoculated parts was collected for RNA analysis. Soil or talc covering the root system was removed by rinsing the roots with water. Total RNA was isolated according to Brusslan and Tobin (1992) and was quantified by spectrophotometry and agarose gel electrophoresis. The RNA samples were treated with DNase I (Promega, Madison, WI, U.S.A.) at 37°C for 45 min, to eliminate traces of contaminating genomic DNA. First-strand cDNA was synthesized using SuperScript II and following the manufacturer's procedure (Invitrogen, Paisley, U.K.). For the PCR amplifications, Finnzymes DyNAzyme DNA polymerase kit (Finnzymes, Espoo, Finland) was used. For the amplification of *PR-1* (At2g19990), *PR-2* (At3g57260), and *PR-5* (At1g75040), the following primer sets were designed: for *PR-1*, 5'-AAG CCT ATC GTC AC ACT CC-3'/5'-AAC CAA GTC GAA CCG AGT C -3'; *PR-2*, 5'-TCA AGG AGC TTA GCC TCA C-3'/5'-CTG ACA CCA TCT CTG TAG C-3'; *PR-5*, 5'-CTC TTC CTC GTG TTC ATC AC-3'/5'-TCT CCG GTT ACA CAT CTA CC-3'. The different cDNA preparations were normalized by parallel amplification of the constitutively expressed gene β -tubulin (At5g12250), using the primer set (*Tub*) 5'-CTC TGA CAC AGT GGT TGA GC-3'/5'-AGA TCA GAG TTG AGC TGA CC-3'. Under our experimental conditions, an exponential increase of the amplification products was observed until 25 amplification cycles ($94^\circ\text{C}/1$ min, $54^\circ\text{C}/1$ min, $72^\circ\text{C}/1$ min and 30 s), so all the amplification reactions were performed under these conditions in order to obtain semiquantitative results. The RT-PCR products were analyzed by 1.5% agarose gel electrophoresis and were blotted and hybridized with the respective digoxigenin-11-rUTP labeled probe.

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