POLITECNICO DI TORINO

Commission of Chemical and Material engineering

Master's degree program
in Chemical and sustainable processes engineering

Master thesis

Arabidopsis K^+ transporter: *At*KUP1 and *At*KUP6 transport activity in Escherichia coli

Advisors

prof. Uozumi Nobuyuki
prof. Bosco Francesca

Candidate

Flavia Carollo

March 2018

“If we knew what it was we were doing,
it would not be called research, would it?”
Albert Einstein

To my parents,
that always support me and
that I love so much

SOMMARIO

Nel laboratorio di Uozumi sensei le ricerche sono focalizzate sull'analisi del trasporto di membrana e trasduzione dei segnali nelle cellule vegetali, animali e nei microrganismi usando l'ingegneria genetica.

La presente ricerca è basata sullo studio dell'attività di due trasportatori di membrana, *At*KUP1 e *At*KUP6, usando *E. coli* LB2003/TO114 come cellula ospite per la loro espressione.
Su questa famiglia di trasportatori sono state condotte diversi tipi di analisi quali l'assorbimento e l'efflusso di K^+. Tali meccanismi, di assorbimento ed efflusso, sono importanti per il mantenimento dell'omeostasi. Questa è importante per la sopravvivenza della cellula in quanto permette di mantenere la concentrazione chimica di molecole e ioni in condizioni stabili. Dal momento che *E. coli* LB2003/TO114 sono delle specie mutate, per il raggiungimento dell'omeostasi necessitano dell'ausilio di trasportatori, quali quelli oggetto di studio del presente elaborato.
Nonostante il fulcro della ricerca sia lo studio dei meccanismi di trasporto del K^+, i test sull'assorbimento sono stati condotti anche su altri ioni come Cs^+, Li^+ e Rb^+. Questi ioni appartengono tutti allo stesso gruppo della tavola periodica (I gruppo) quindi hanno la stessa carica. Se un trasportatore di membrana è in grado di assorbire K^+ ci sono buone possibilità che sia in grado di assorbire anche gli ioni elencati sopra.

Le membrane definiscono i confini esterni delle cellule e regolano il "traffico" molecolare all'interno della cellula; nelle cellule eucariotiche dividono lo spazio intracellulare in compartimenti.
La membrana plasmatica è composta da lipidi e proteine che formano una barriera sottile, resistente e idrofobica. É una barriera per il libero passaggio di ioni inorganici e la maggior parte dei composti polari. Secondo il modello di S. J. Singer e G. L. Nicholson, la membrana viene descritta come un "*mosaico fluido*". Secondo tale modello, la membrana è allo stato di liquido-cristallino ed include una vasta gamma di proteine specializzate che catalizzano o promuovono diversi processi cellulari. Tali proteine, grazie alla fluidità della componente lipidica, hanno un alto grado di mobilità.
In Fig.1 è possibile vedere un esempio di membrana plasmatica appartenente ad una cellula animale. È una struttura dinamica in continuo rimodellamento per permettere alla cellula di rispondere ai cambiamenti e alle diverse condizioni ambientali.
Per vivere e crescere le cellule hanno bisogno di scambiare molecole. A causa del carattere idrofobico della membrana, l'assorbimento di molecole polari non è semplice. Sono necessari, quindi, dei trasportatori di membrana che permettono il passaggio selettivo di alcune sostanze. Canali e trasportatori, che mediano il trasporto ionico e di soluti attraverso la membrana, garantiscono una resistenza della cellula a cambiamenti ambientali e stress legati all'alta pressione osmotica.

Ioni inorganici come Na^+, K^+, Ca^{2+}, Cl^- e H^+ sono i più abbondanti soluti che circondano le cellule e il loro passaggio attraverso la membrana plasmatica ha un ruolo importante per molti processi biologici, come la produzione di ATP.
Ogni cellula vivente possiede a livello della membrana plasmatica una differenza di potenziale (ddp) chiamato potenziale di membrana. Quest'ultimo è definito come la differenza di potenziale elettrico tra l'interno e l'esterno della cellula ovvero la differenza tra numero di cariche positive e negative in prossimità della membrana. È necessario mantenere un equilibrio tra carica elettrica interna ed esterna.

Vi sono due principali classi di proteine di trasporto: trasportatori e canali. La principale differenza tra i due è il meccanismo con cui trasportano e selezionano certi soluti e non altri.
I **trasportatori** permettono il passaggio di molecole e ioni che si adattano ad un corrispondente sito di legame della proteina. Dopo aver legato il soluto, il trasportatore modifica la sua conformazione e trasferisce una molecola per volta sull'altro lato della membrana. I trasportatori funzionano più come una porta girevole che come una porta aperta. ("Zanichelli, L'essenziale di Biologia molecolare della cellula, Bologna, 2011"). Come per l'enzima e il suo substrato, vi è un'associazione specifica tra un trasportatore e il suo soluto.
I **canali**, invece, permettono il passaggio dei soluti in relazione alla dimensione e alla carica elettrica. Se il canale è aperto, una molecola o ione sufficientemente piccolo e dotato di carica adatta può infilarsi in questa specie di botola e attraversarla ("Zanichelli, L'essenziale di Biologia molecolare della cellula, Bologna, 2011").
I trasportatori a loro volta si suddividono in attivi e passivi (Fig. 4).
I *trasportatori passivi* semplicemente facilitano la diffusione che avviene spontaneamente grazie ad un gradiente di concentrazione. Non vi è alcuna spesa di energia in questo caso.
I tr*asportatori attivi* guidano i soluti attraverso la membrana contro un gradiente di concentrazione. In questo caso vi è un consumo di energia. I trasportatori attivi primari usano l'energia fornita da una reazione chimica mentre i secondari accoppiano il trasporto "a valle" di un soluto con quello "a monte" di un altro soluto.

Il potassio (K^+) è un macronutriente essenziale per la crescita e lo sviluppo delle piante. È il catione più abbondante nelle cellule vegetali. L'assorbimento di K^+ può essere influenzato da stress ambientali quali salinità, tossicità di metalli e siccità ma questi problemi possono essere superati incrementando il rifornimento di K^+. Il potassio è importante per il mantenimento dell'apparato fotosintetico e una sua carenza riduce l'attività fotosintetica e il contenuto di clorofilla. Per un rapido assorbimento del K^+ sono necessarie delle proteine di membrana. Una vasta varietà di trasportatori sono già stati identificati a livello molecolare, dimostrando la complessa natura del trasporto di K^+. Questi includono trasportatori ad alta affinità e canali ionici.

Il cesio (Cs) è un metallo alcalino appartenente al primo gruppo della tavola periodica. A differenza del potassio, non è un elemento essenziale per le cellule viventi. Ha guadagnato attenzione a livello mondiale a causa della grande quantità di cesio radioattivo (^{134}Cs e ^{127}Cs) rilasciato in seguito agli incidenti nucleari di Chernobyl nel 1986 e Fukushima nel 2011. Sono stati avviati vari studi sull'impatto ambientale della contaminazione di Cs^+. Molte ricerche sono focalizzate sull'uso di microrganismi altamente tolleranti verso il Cs^+ al fine di utilizzarli per tentativi di decontaminazione e bio-risanamento.

Il cesio è una sostanza tossica per microrganismi, cellule animali e vegetali. A causa delle caratteristiche chimiche simili con il potassio, è stato proposto che il Cs^+ sia in grado di entrare nelle cellule attraverso i trasportatori del potassio.

MATERIALI

Nella ricerca come sistema di espressione delle proteine ricombinanti è stata usata una cellula batterica, *Escherichia coli*. È un batterio Gram-negativo. È costituito da una membrana esterna con funzione protettiva e da una membrana plasmatica interna, che racchiude citoplasma e nucleotidi. Tra le due membrane vi è la parete cellulare, un sottile ma resistente strato di polimeri, i peptidoglicani, che conferiscono rigidità e forma alla cellula ("W.H.Freeman & Co Ltd; 6th edition, 2013").

Per gli esperimenti sono stati usati due diversi tipi di mutanti:

- ***E. coli* LB2003**: L'*E. coli* ha tre principali sistemi di assorbimento per il K^+ (Kdp, Trk e Kup) espressi sulla membrana interna. *E. coli* LB2003 è un mutante che non presenta questi tre trasportatori, quindi, l'assorbimento del potassio è molto basso. Inserendo dei trasportatori ricavati da un'altra cellula, nel caso della ricerca da una cellula vegetale, l'assorbimento aumenta notevolmente e può così essere misurata facilmente la loro attività (Fig. 7).
- ***E. coli* TO114:** L'*E. coli* ha tre principali sistemi di efflusso per il K^+ (ChaA, NhaB e NhaA) espressi sulla membrana interna. L'efflusso dei trasportatori dell'*A. thaliana* può essere misurato nell'*E. coli* TO114 che è un mutante privato dei tre sistemi di efflusso (Fig. 8).

Le cellule vegetali sono cellule eucariotiche, infatti, il DNA è racchiuso all'interno di un nucleo. La più grande differenza tra cellule animale e vegetali è la presenza della parete cellulare e degli organelli fotosintetici nelle ultime.
Questa è costituita da cellulosa e dà supporto e rigidità alla cellula. Vi sono numerosi organelli con diverse funzioni necessarie per la sopravvivenza e normali operazioni delle cellule come produzione di ormoni, enzimi e tutte le altre attività metaboliche.
A.Thaliana è una piccola pianta diventata un sistema modello per molte ricerche in ambito biologico. Possiede un piccolo genoma e l'intero ciclo vitale è completato in 6 settimane. Le piantine possono crescere in capsule Petri o in vasi posti in serre o sotto luci fluorescenti in laboratorio. Il genoma è organizzato in 5 cromosomi e contiene 2000 geni.
Possiede geni omologhi all' *E. coli.* Due geni sono definito omologhi quando le sequenze del DNA derivano da un'origine comune e posso avere o meno la stessa funzione.
Per questo motivo l'*E. coli* viene usato come cellula ospite nello studio dei trasportatori di membrana *At*KUP1e *At*KUP6 appartenenti all'*A. thaliana.*

*At*KUP1 e *At*KUP6 sono due sistemi di trasporto appartenenti ad una delle sei famiglie di trasportatori cationici dell'*Arabidopsis thaliana* permeabili al K^+: canali tipo Shaker K^+, canali 'two-pore' K^+, canali Cyclinucleotide-gated, antiporto K^+/H^+, trasportatori *KUP/HAK/KT* e trasportatori HKT.

I test sono stati condotti su terreni ricchi in potassio, sia liquidi che solidi. L'unica differenza tra questi è l'aggiunta di 20 [gr/L] di agar nel caso di terreno solido.

METODI

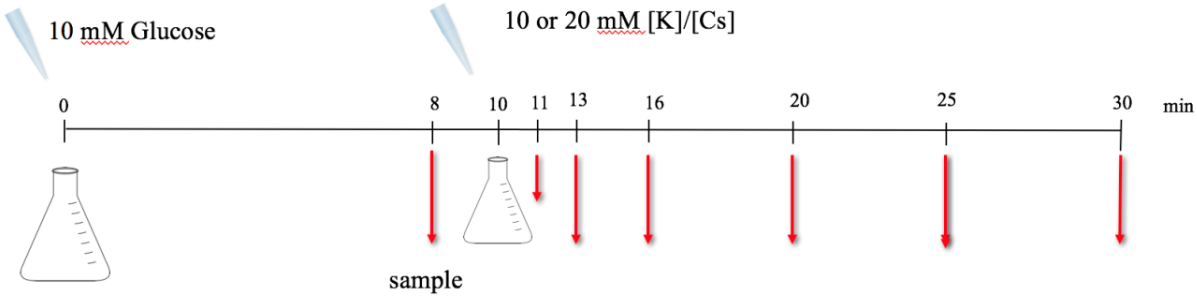
Sono stati condotti diversi tipi di esperimenti per analizzare il meccanismo di funzionamento dei trasportatori di membrana descritti sopra.

Il primo esperimento condotto è stato il **complementation test**. Lo scopo del test è stato quello di osservare se i trasportatori inseriti nella cellula ospite sono in grado di garantirne la crescita. La cellula ospite utilizzata, come detto prima, è *E. coli* LB2003 e il test viene condotto confrontando la crescita di 4 diversi campioni: LB2003+EV (vettore vuoto), LB2003+*At*KUP1, LB2003+*At*KUP6 e LB2003+*Ec*KUP. Dove le sigle si riferiscono: LB2003 alla cellula ospite e EV/ *At*KUP1/ *At*KUP6/ *Ec*KUP al vettore ospitato.
Il terreno usato è un LBK30, ovvero un terreno ricco in potassio.
L'esperimento si sviluppa in tre giorni. Si inizia con la fase preparatoria durante la quale si prepara la pre-coltura la quale viene incubata a 30°C per 24-48 ore. Trascorso il tempo necessario è possibile fare un'analisi qualitativa della crescita la quale sarà diversa a seconda del campione.
La crescita viene osservata visivamente nel caso di terreno solido mentre nel caso di terreno liquido viene osservata misurando la densità ottica a 600 nm (OD600).
Lo scopo dell'esperimento è, inoltre, quello di osservare come viene influenzata e cambia la crescita dei vari campioni al variare della concentrazione di K^+ nel terreno di coltura.
Si prelevano le colture cresciute sul terreno ricco e si inoculano in terreni a diversa concentrazione di potassio K1, K5, K10 e K20 (1mM, 5mM, 10 mM e 20 mM). Si incubano nuovamente le colture a 30°C per 24-48 ore. Lo stesso esperimento è stato ripetuto tre volte.

L'**assorbimento** del K^+, Cs^+, Rb^+ e Li^+ è stato testato in *E. coli* LB2003. Per avviare l'esperimento è necessario inoculare il mutante contenente il vettore d'interesse in un terreno ricco in potassio (KLB 300). L'inoculo viene prelevato da uno stock di glicerolo contenete le cellule, precedentemente preparato e conservato a -80°C.
L'esperimento inizia con la preparazione dei terreni di coltura per la pre-coltura e per la coltura. Questi sono dei terreni ricchi in KCl, quindi con un'alta concentrazione di potassio. Vengono preparati in delle beute (10 mL di terreno pre-coltura; 200 mL coltura) sottoposte ad autoclave (sterilizzazione termica) prima di essere usati per l'esperimento.
Le specie vengono inoculate ed incubate a 30°C per circa 7 ore.
Trascorso il tempo necessario, viene misurata la densità ottica (OD578) per controllare la crescita cellulare. Maggiore densità ottica equivale a maggiore quantità cellulare.
Per ottenere dei risultati validi è importante lavorare con cellule che si trovano nella fase di crescita esponenziale.
Si procede con la coltura la quale, a sua volta, viene incubata a 30°C per una notte.
Trascorso il tempo necessario, la coltura viene trasferita in una provetta e centrifugata in modo da ottenere un pellet contenente le cellule di interesse.
Questo viene risospeso in Tris HCl (pH 8,0) in modo da avere OD578=30.
Dopo aver aggiunto 100 µL di EDTA (acido etilendiamminotetraacetico), la soluzione viene nuovamente incubata ma a 37°C per 30 minuti.

L'EDTA viene usata per cattura tutti gli ioni che non interessano ai fini dell'esperimento.
Trascorsi i 30 minuti, il pellet viene recuperato e sottoposto a "lavaggio" in modo da eliminare il terreno di coltura residuo. L'operazione viene svolta usando come agente di lavaggio 200 mM HEPES NaOH pH 7,5.
Terminata la fase di lavaggio, la soluzione di interesse viene trasferita in delle beute da 50 mL, regolando la densità ottica in modo che sia OD578=3.
I campioni prelevati verranno inseriti in degli Eppendorf tube (1,5 mL) contenenti olio siliconico che servirà nella fase di centrifugazione per separare il pellet dal surnatante.
Il cuore dell'esperimento consiste:

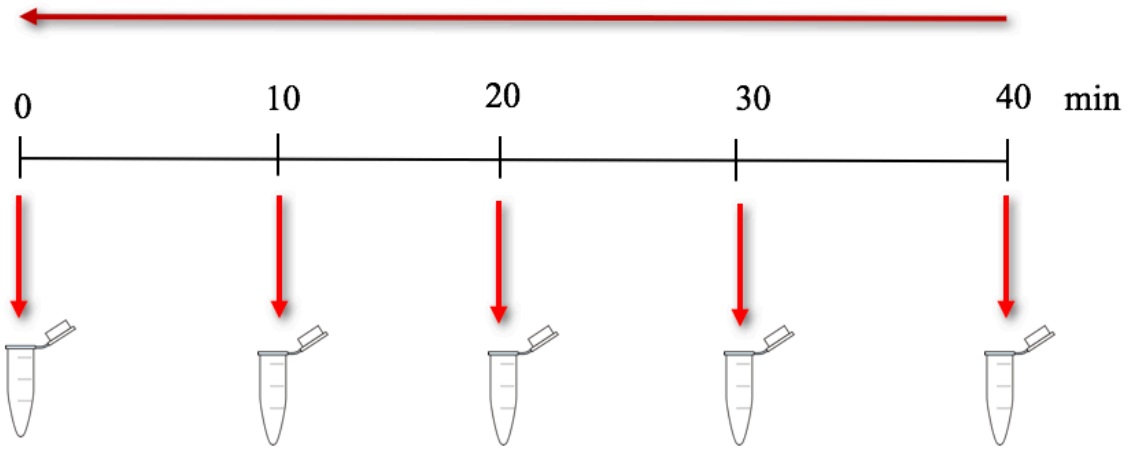
- Inizio (minuto 0): aggiunta di 600 µL 0,5 M di glucosio nella beuta;
- Primo campione (minuto 8): prelevare ed inserire 1 mL della sospensione cellulare nell' Eppendorf tube e centrifugarlo immediatamente a 15000 rpm per 1 minuto;
- Dopo 10 minuti: aggiunta di 120 µL di 10 o 20 mM [K]/[Cs];
- Prelevare i diversi campioni fino a 30 minuti.



Il pellet viene recuperato e risospeso aggiungendo acqua distillata. Vengono aggiunti 500 µL di 10% TCA (acido tricloroacetico) e i campioni vengono posti a 100°C per indurre la morte delle cellule di *E. coli*.
I campioni vengono centrifugati e il surnatante ottenuto verrà usato nell'analisi della concentrazione di potassio/cesio fatta tramite AAS.

AAS (Atomic Absopition Spectrometry) è una tecnica usata per misurare la quantità di elementi chimici presenti nei campioni analizzati. Viene misurata la radiazione assorbita dagli elementi chimici di interesse.

In parallelo all'assorbimento, è stato analizzato anche l'**efflusso** del potassio usando come mutante *E. coli* TO114. L'efflusso, al contrario dell'assorbimento, è il passaggio del K^+ dall'interno all'esterno della membrana plasmatica.
Il terreno di coltura è un terreno ricco in potassio LBK300 e viene preparato in delle beute da 100 mL sottoposte a sterilizzazione termica.
Come per l'uptake, è necessario preparare una pre-coltura da incubare a 30°C per una notte.
Questa servirà per la coltura principale preparata in delle beute che vengono a loro volta incubate a 30°C fino a quando l'OD660=0,6-0,7.
Nel momento in cui il target viene raggiunto, la coltura viene trasferita in provette da 50 mL e centrifugata. Il pellet ottenuto viene risospeso con KCl 0,4 M.
Il corpo dell'esperimento consiste nell'inserire, all'interno del buffer di reazione, 16 µL della sospensione cellulare ogni 10 minuti.



Allo scadere del tempo i campioni vengono centrifugati per un minuto. A questo punto, le operazioni svolte sono analoghe a quelle svolte nell'esperimento precedente.
Questo esperimento è stato svolto utilizzando diverse condizioni di reazione, ovvero buffer di reazione aventi diversi pH (5, 6, 7, 7.5, 8, 9). Non sono stati ottenuti risultati postivi con la maggior parte di essi. Solo con **HEPES NaOH Ph 8,0- 0,4 M NaCl** (Fig. 35) è possibile osservare una leggera attività di efflusso da parte di *At*KUP1e *At*KUP6 se messa a confronto con quella dell'EV.

RISULTATI

Dall'analisi dei dati ottenuti con il **complementation test** su terreno solido si è potuto concludere che entrambi *At*KUP1 e *At*KUP6 permettono la crescita della specie (*E. coli* LB2003) in condizioni di alta concentrazione di potassio mentre la crescita si riduce considerevolmente a bassa concentrazione. *At*KUP6 ha una crescita più bassa rispetto ad *At*KUP1 a basse concentrazioni.
Per quanto concerne il terreno liquido, oltre ad analizzare la crescita al variare della concentrazione di potassio, sono stati fatti test aggiungendo nel terreno di coltura diverse concentrazioni di cesio.
Per il test in cui è stata variata la concentrazione di potassio, i risultati rispecchiano quelli ottenuti con il terreno solido.
Nei test condotti aggiungendo cesio si può notare che a basse concentrazioni non influisce negativamente sulla crescita. Di contro aumentandone la concentrazione la crescita decresce. Si deduce, quindi, che probabilmente il cesio ad alte concentrazioni inibisce la crescita delle specie (LB2003+EV, LB2003+*At*KUP1, LB2003+*At*KUP6 e LB2003+*Ec*KUP) analizzate.

Dopo aver analizzato entrambi i meccanismi di **assorbimento** ed efflusso al variare delle condizioni ambientali, si può concludere affermando che:

- *At*KUP1 ha un'elevata attiva di assorbimento ed è in grado di assorbire diversi ioni, non solo K^+. In contrasto rispetto all'assorbimento, l'attività di efflusso è molto limitata e si osserva solo usando buffer a pH 8. La crescita di *E. coli* LB2003 è molto lenta ma è stato osservato che con l'espressione di un vettore contente *At*KUP1 e con

elevate concentrazioni di K^+ nel terreno di coltura, la velocità di crescita aumenta notevolmente.

- *At*KUP6 non possiede né un'elevata attività di assorbimento né di efflusso. Non è in grado di assorbire altri ioni all'infuori del K^+. Una lieve attività di efflusso può essere osservata a tre diversi valori di pH. In merito al complementation test, è in grado di velocizzare la crescita di *E. coli* LB2003 ma risulta inferiore rispetto alla crescita di *E. coli* LB2003 +*At*KUP1.

ABSTRACT

Potassium (K) is an essential macronutrient in higher plants. It's an obligatory component of living cells to grow and to keep themselves alive. K^+ is an absolute requirement for many cellular functions, such as osmotic regulation, protein synthesis and enzyme activation. The accumulation of K^+ by the plant root symplast imposes a substantial energetic cost and requires specialized transport systems.
Transmembrane movement of K^+ is catalyzed by channel and transporter proteins, and energized by the negative membrane potential of plant cells.
Arabidopsis has six major K^+ uptake system: Shaker-type K^+ channels, 'two pore' K^+ channels, cyclic-nucleotide-gated channels, putative K^+/ H^+ antiporters, KUP/HAK/KT (high affinity K^+ symporter family) and HKT (high affinity K^+ transporters). *At*KUP1 and *At*KUP6 belong to the KUP/HAK/KT transporters family. KUP is a high affinity K^+ transport system and it also transports Rb^+ and Cs^+.
Caesium (Cs), in contrast to K^+, is not an essential element for living cells although it is chemically similar to potassium. Cs is toxic to plants and it's a competitive inhibitor for potassium uptake as an analogous element.
It has gained special world-wide attentions due to the release of considerably large amounts of radioactive caesium (^{134}Cs and ^{127}Cs) after the power plant accidents in Chernobyl in 1986 and Fukushima in 2011. This has led to studies of the environmental impact of Cs^+ contamination, decontamination attempts and bioremediation efforts as the research of microorganisms highly tolerant to Cs^+ ("Tanudjaja, Hoshi, Su, Hamamoto, Uozumi, 2017").

The research is based on the study of *At*KUP1 and *At*KUP6 transport activity using *Escherichia coli* LB2003 and TO114 as host cells for protein expression system.
First of all, LB2003 and TO114 (*E. coli* mutants) were transformed with plasmids containing *At*KUP1 gene and the *At*KUP6 gene. The growth of *E. coli* mutants was tested in a solid synthetic medium, and at a later stage in a liquid synthetic medium, using different concentrations of potassium (1, 5, 10 and 20 [mM]).
At the same time *E. coli* mutants were transformed with a plasmid containing an empty vector (EV).
The growth of the strains was compared to the growth of an empty vector (EV, negative control) and *Ec*Kup (positive control).
EV should give a negative control because of the lack of transporters and it should show very low to no growth.
*Ec*Kup (*Escherichia coli* K^+ uptake) is the cell without transporters lack so in contrast to the negative control it should show a very high growth.
The uptake test was performed for EV, *At*KUP1 and *At*KUP6 by addition of 120 µl of 10 [mM] K^+, 20 [mM] Cs^+, 20 [mM] Rb^+ and 20 [mM] Li^+ to the medium.
In parallel the efflux test for the same strains was performed using buffer with different pH (citric acid-Na_2HPO_4 pH 5.0, MES Tris pH 6.0- 0.4M NaCl, HEPES NaOH pH 7.0 - 0.4M NaCl, HEPES NaOH pH 7.5 - 0.4M NaCl, HEPES NaOH pH 8.0 - 0.4M NaCl, CHES NaOH pH 9.0 – 0.4M NaCl).

The intracellular concentrations of ions were measured by Atomic Absorption Spectroscopy (AAS).

*At*KUP1 and *At*KUP6 are two members of the KUP (K^+ high affinity) family.
As has been mentioned before, *E. coli* strain LB2003 lacks TrK, Kup and Kdp K uptake transporters. Since Potassium is an essential nutrient for the cell growth it's necessary it can be absorbed well by the cells. To overcome the lack *At*KUP1 and *At*KUP6 were inoculated in the mutant to allow the K^+ uptake.
Several assays were performed to understand if they can actually complement a defect in potassium uptake activity in an *Escherichia coli* mutant (LB2003).
Growth test in solid synthetic medium of the strains shows that with an increase of the K^+ concentration, the growth of *At*KUP1 and *At*KUP6 increase. That means they can complement the lack of transporters.
EV (empty vector) didn't grow with low K^+ concentration (1 [mM]) as expected. There aren't remarkable differences between *At*KUP1, *At*KUP6 and *Ec*KUP in the medium with 10 and 20 [mM] K^+.
The values of complementation test with the liquid synthetic medium were collected after 8 hours of incubation and after 24 hours. As in the solid medium, the assay was performed with different K^+ concentrations (1, 5 10 and 20 [mM] K^+).
The K^+ levels of 1 [mM] K^+ did not show significant difference between the different strains. However, in the medium with 5, 10 and 20 mM K^+, the LB2003 expressing AtKUP1 has a faster growth rate compared with EV and *At*KUP6.
The cells expressing *At*KUP6 grew faster than the cells with EV only with 20 [mM] of K^+.

The K^+ uptake assay shows positive data for *At*KUP1.
As shown in Fig. A, LB2003 transformed with *At*KUP1 (orange curve) had a high uptake activity for K^+ as well as with Cs^+.
On another hand, LB2003 transformed with *At*KUP6 (grey curve) doesn't show positive data for the Cs^+/ K^+ uptake. That means *At*KUP1 is able to uptake K^+ indeed the cell can grow well but *At*KUP6 doesn't uptake K^+ so the growth of the cell is not high. Indeed, it is almost the same of the EV growth, there aren't significant differences between EV (blue) and *At*KUP6 (grey).

From the efflux assays the efflux activities by KUP1 and KUP6 is very low.
Only weak efflux activity by KUP6 was observed only in the HEPES NaOH pH 8.0 - 0.4M NaCl for *At*KUP6/KUP1.
Fig. B shows a weak K^+efflux (K^+ goes from the inner to the outer part of the plasma membrane) activity even if the gap between *At*KUP6/KUP1 and EV is no so high.

From the results above can be said AtKUP1 has a K^+ uptake/efflux and Cs^+ uptake activity while AtKUP6 has only a K^+ efflux activity.
Competition between K^+ and Cs^+ for K^+ association sites on vital proteins like *At*KUP1 may cause the cytotoxicity of Cs^+.

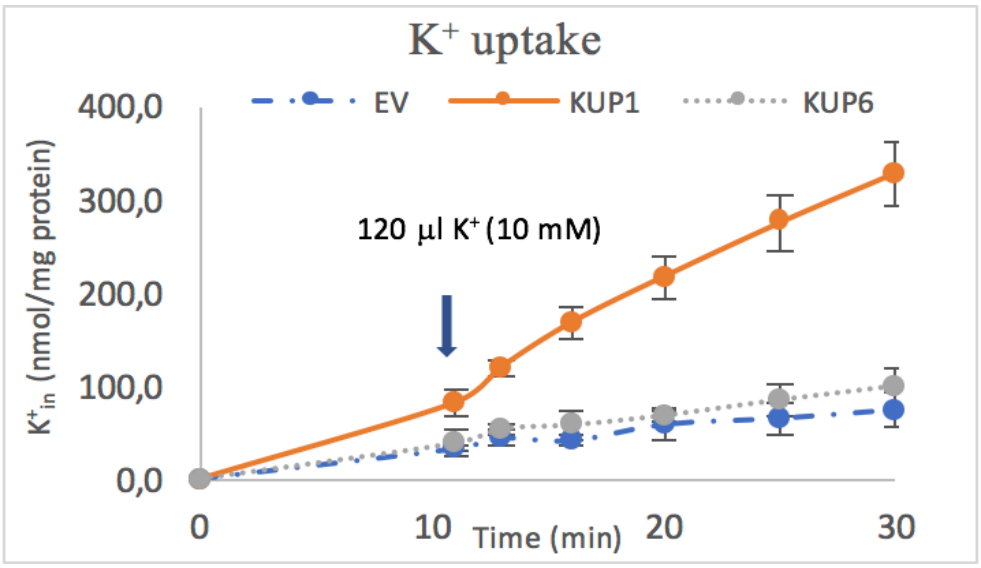


Figure A. K^+ uptake measurement of *E.coli* expressing *At*KUP1, *At*KUP6 and EV.

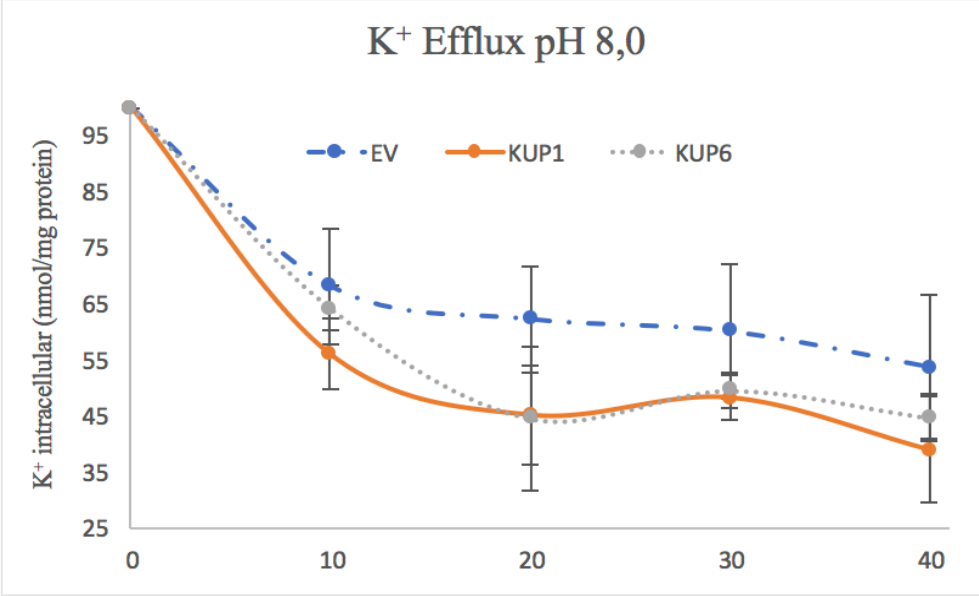


Figure B. K^+ efflux measurement of E.coli expressing AtKUP1, AtKUP6 and EV.

D

Table of Contents

SOMMARIO I

ABSTRACT ..... A

1. INTRODUCTION 1

1.1 WHAT IS THE PLASMA MEMBRANE? 1

1.2 ESCHERICHIA COLI 6

1.3 WHAT IS A PLANT CELL? 9

1.3.1 ARABIDOPSIS THALIANA 9

1.5 POTASSIUM 12

1.6 CAESIUM 13

1.7 A BRIEF DESCRIPTION OF DNA RECOMBINATION TECHNOLOGY 15

2. PURPOSE OF THE THESIS 17

3. MATERIALS AND METHODS 19

3.1 MATERIALS 19

3.1.1 PLANT MATERIALS 19

3.1.2 BACTERIA 19

3.1.3 SOLID AND LIQUID MEDIUM 19

3.1.4 GLYCEROL STOCK 20

3.2 METHODS 21

3.2.1 TRANSFORMATION WITH HEAT SHOCK 21

3.2.2 COMPLEMENTATION TEST IN SOLID/LIQUID SYNTHETIC MEDIUM 22

3.2.3 CATION UPTAKE EXPERIMENT 24

3.2.4 POTASSIUM EFFLUX EXPERIMENT 30

3.2.5 AAS ION MEASUREMENT 33

3.2.6 BCA PROTEIN ASSAY 35

4. RESULTS 37

4.1 TRANSFORMATION WITH HEAT SHOCK 37

4.2 COMPLEMENTATION TEST IN SOLID/LIQUID SYNTHETIC MEDIUM 37

4.2.1 SOLID MEDIUM 37

4.2.2 LIQUID MEDIUM 41

4.3 CATION UPTAKE EXPERIMENT 43

4.4 POTASSIUM EFFLUX EXPERIMENT 46

5. CONCLUSIONS 51

6. REFERENCES 53

LIST OF FIGURES **57**
LIST OF TABLES **58**
ACKNOWLEDGEMENTS **61**

1. Introduction

1.1 What is the Plasma Membrane?

Every organism is made by living cells. A living cell is a system of molecules that is able to reproduce itself and it is surrounded by a membrane, the *Plasma Membrane.* ("Zanichelli, Bologna, 2011").
Membranes define the external boundaries of cells and regulate the molecular traffic across that boundary; in the eukaryotic cells, they divide the internal space into discrete compartments to segregate processes and components. ("W.H.Freeman & Co Ltd, 6th edition, 2013").
The Plasma membrane is composed of lipid and protein molecules that form a thin, tough, pliable, hydrophobic barrier around the cell. The membrane is a barrier to the free passage of inorganic ions and most other polar compounds.
It's not a passive barrier indeed it include an array of proteins specialized for catalysing or promoting different cellular processes.

Membrane proteins function are:

1. to anchor the membrane to the cytoskeleton and/or cell wall,
2. as receptors/transducers for compartmentalized signals,
3. as enzymes for specific reactions, such as energy transduction processes in mitochondria and chloroplasts,
4. to transport specific solutes across membranes.

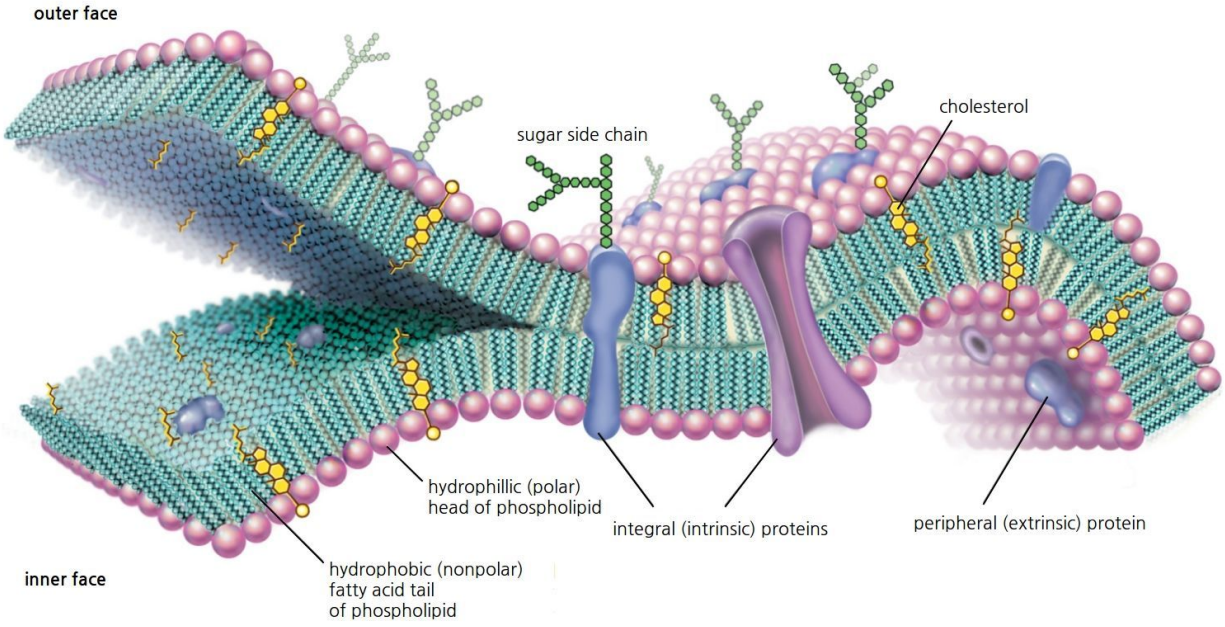


Figure 1. Animal cell plasma membrane (Encyclopaedia Britannica/UIG/Getty Images)

Fig. 1 show a membrane and how is a dynamic structure that is continuously remodeled to allow the animal cell (in this case) to respond to developmental signals and environmental

conditions. This remodeling occurs over minutes to months, and is supported by complex trafficking pathways that deliver lipids and proteins to and from cellular membranes.
To live and grow a cell needs to exchange molecules. Since the inner part of the double lipid layer is hydrophobic (fig. 2), the membrane doesn't allow the cross of almost all the water-soluble molecules. Nevertheless, some of the water-soluble molecules have to cross the membrane because are essential nutrient for the cells as sugars and amino acids.
Moreover, waste products of metabolic processes have to live the cells as CO_2. ("Zanichelli, Bologna, 2011")

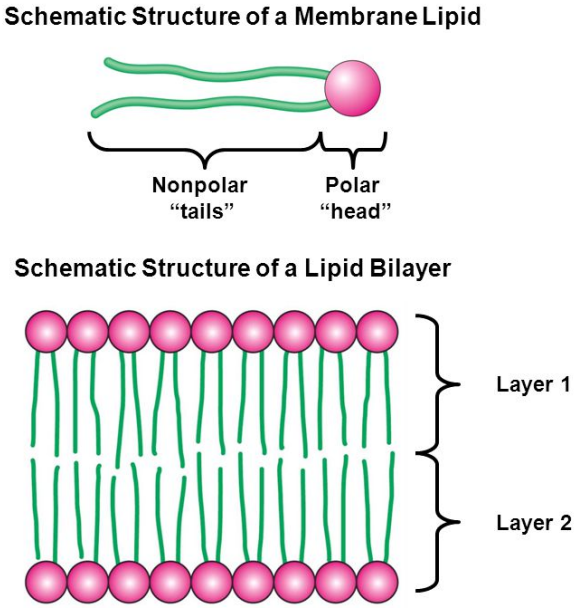


Figure 2. Lipid bilayer (http://images.slideplayer.com/17/5357157/slides/slide_4.jpg)

How can these molecules cross the hydrophobic double-layer?
Compounds like CO_2 and O_2 can cross easily by diffusion the double-layer but substances like ions need transport protein to cross the membrane.

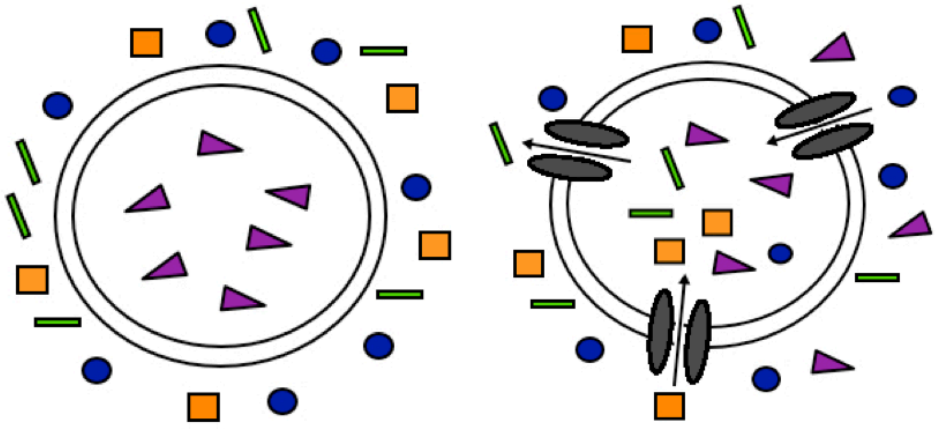


Figure 3. Synthetic double lipidic layer without proteins (left) and plasma membrane (right).

Living organisms such as plants, animals and microorganisms contain various kinds of elements and nutrients, which are actually required within the biological membrane.

The organism selectively takes up molecule based on factors such as type, timing and age and the needs of the tissues and organs.
Channels and transporters, which mediate the transport of ion and solute across the biological membrane provide resistance to environmental changes and stresses associated with salt and high osmotic pressure (typically salt damage and drought).
The plasma membrane can be cross by a lot of different molecules. Every protein is selective so only a specific set of solutes can enter inside the membrane. The internal ionic composition of living cells is different than the fluid in the external part. This difference is very important and it has a key role in the sustainability and proper functioning of the cell.
Inorganic ions like Na^+, K^+, Ca^{2+}, Cl^- and H^+ are the most abundant solute that surround the cells and their transit through the plasma membrane has an important role for biological process, like ATP production. ("Zanichelli, Bologna, 2011")

Na^+ is the most abundant ion in the outer part of the cell while K^+ is the most abundant in the inner part.
Since a cell isn't torn because of the electric force, it's necessary an equilibrium between the inner and outer electrical charge.
The positive charge should be the same of the negative charge even if there are little excess of positive or negative charge near the membrane.
The outer high concentration of Na^+ is balanced by extracellular Cl^-, while the inner high concentration of K^+ is balanced by different intracellular ion.

The different intracellular and extracellular ion distribution depends on the protein activity, that are involved in the transport, and on the permeability of the double layer.
The hydrophobic inner part of the double layer is a barrier for the crossing of the water-soluble molecules and ions. The hydrophobic molecules stay away from the water.

Every molecule can cross the plasma membrane if it has enough time. The diffusivity velocity of a substance changes based on the dimensions of the molecules and the solubility.
If a molecule is little and soluble in fats, its diffusion shall be quick through the lipid membrane.
Ions and the bigger molecules without charge cannot cross the membrane by diffusion so they need the transport membrane proteins.
A molecule crosses the membrane quicker if it is small and if it has weak interaction with water. Most of the molecules cannot cross the membrane because they are too big and polar.

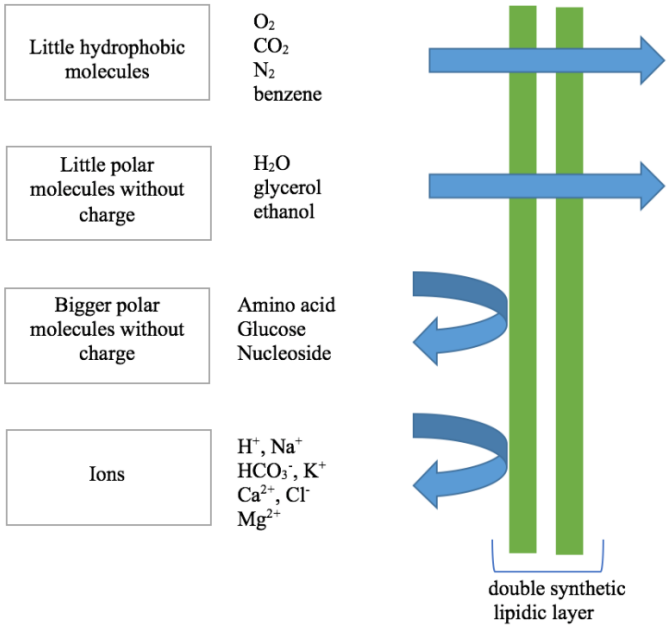


Figure 4. Double layer permeability based on the molecules dimensions. (Zanichelli, 2011 with modifications)

There are two very broad categories of transporters:

- ***Carriers*** allow the cross of ions or molecules that are able to create a link with the binding site of the protein. After the transporter links the solute, it changes its shape to uptake the molecule/ion in the inner part. They have a high selectivity. This process resembles an enzyme-substrate reaction, and in many ways carriers behave like enzymes. In this case, in contrast to the enzyme-substrate reaction, the transported solute in not covalently modified by the carrier protein. ("Molecular Biology of the Cell, 4th edition").
- ***Channel*** selects solutes mostly according to the size and the electric charge. If the ion or molecule is enough small and has a particular electric charge, it can easily cross the membrane through the channel. They can show three important properties: they conduct ions rapidly, many ions channels are highly selective (only certain ion species flow), their function is regulated by gating (ion conduction is turned on and off in response to specific environmental stimuli). ("R. MacKinnon, 2004").

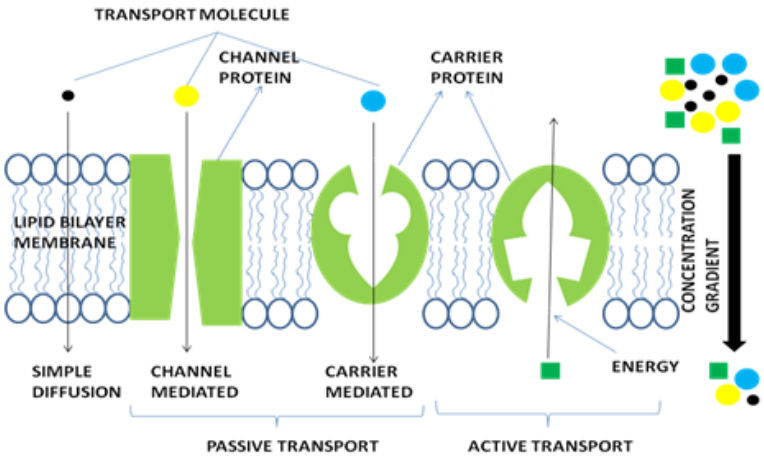


Figure 5. Passive or Active Transport, Channel or Carrier Protein (Garland Science 2014)

This classification is the main distinction among transporters even though there are other differences.
Since the research is focus on carrier, a brief explanation of their main properties will be given below.

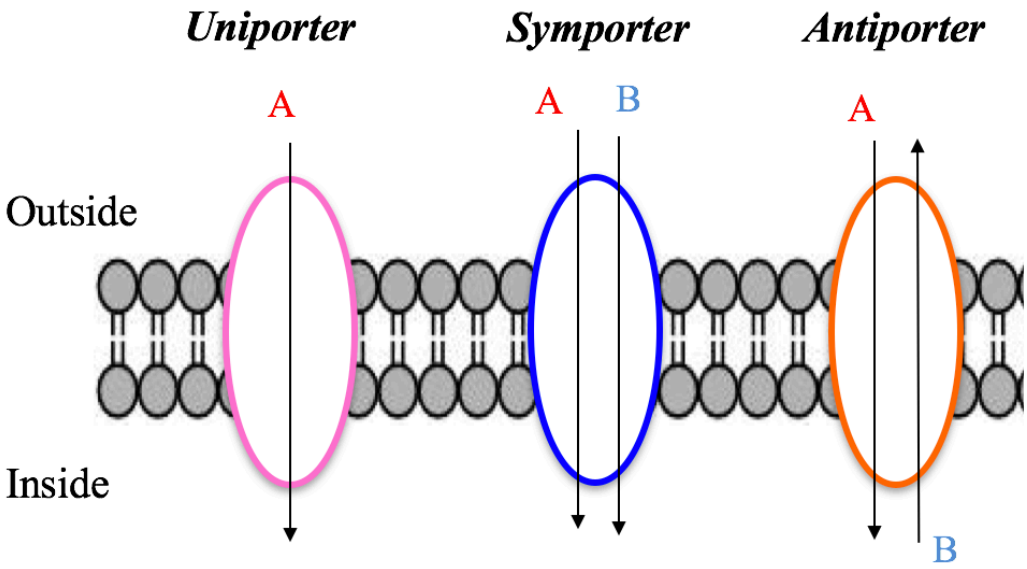


Figure 6. Carriers classification (Zanichelli, 2011 with modifications)

How it shows in fig.6 there are three family of carriers:

- The uniporter family allow the membrane cross of only one solute;
- The symport family allow the membrane cross of two solute in the same direction;
- The antiporter family allow the membrane cross of two solute but in opposite direction.

An important difference is between active and passive transporters (fig. 4). Passive transporters simply facilitate diffusion down a concentration gradient.

Active transporters can drive substrates across the membrane against a concentration gradient, primary active transporters using energy provided by a chemical reaction and secondary active transporters coupling uphill transport of one substrate with the downhill transport of another.

The research involves analysis of the critically important channel transporter system at the genetic and molecular level. With recent advances in structural analysis of soluble proteins, the focus has shifted to the study of more elusive biological membrane proteins.
Although the genetics of the membrane transport system are established, their structures, functions and roles remain to be elucidated.
In particular, malfunction of membrane transport proteins confers some hereditary disease, and hypersensitive response to abiotic and biotic stress.
Analysis and applied research in this area will become important in the future.
The research extends to the biomolecular study of organism, as well as intracellular genes and other mechanisms that regulate the membrane transport system in accordance with changes in the external environment.

1.2 Escherichia coli

It is a bacteria that is located also in the human stomach (harmless inhabitant). It's a Gram-negative bacillus with a rod-shaped, measures only 2 μm long and less than 1 μm in diameter.
It has an inner plasma membrane, that contains the cytoplasm and the nucleoid, and an outer membrane. Between the two membranes is the cell wall, a strong layer of polymers called peptidoglycans, which gives the cell its shape and rigidity. ("W.H.Freeman & Co Ltd; 6th edition, 2013").
The complete genome contains a single circular DNA molecule composed of 4,639,221 bp.
It can reproduce itself quickly by scission, creating colonies of clones on agar plates (solid medium) and it's also able to grow on liquid medium.
It uses wildly for experiments because it produces a high number of daughter cells.
It's haploid meaning it has one round chromosome.
The most important properties are: Easy to culture and manipulate and fast duplication time.
The advantages of using *E. coli* as the host organism are well know: (i) it has unparalleled fast growth, (ii) high cell density are easily achieved, (iii) rich complex media can be made from readily available and inexpensive components and (iv) transformation with exogenous DNA is fast and easy. ("Rosano and Ceccarelli, 2014").
Plasmid transformation of *E. coli* can be performed in as little as 5 min ("Pope and Kent, 1996").
E. coli was used in this research because it owes several natural plasmids.

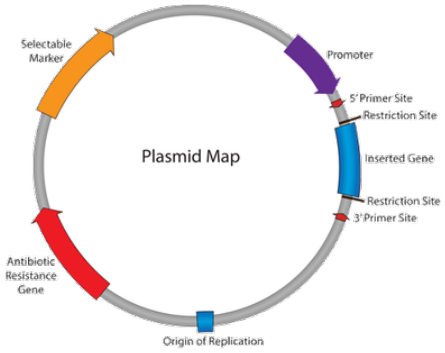


Figura 7. (ddgene.org/plasmids-101-what-is-a-plasmid)

A **plasmid** is a small circular DNA molecule within a cell and it's naturally exchanged among organisms. They are excellent DNA delivery vectors.

Circular DNA is well suited to incorporate extra DNA sequences because it can be cut open without falling apart and then snap back together as soon as new DNA has been incorporated.

They can be copied several times regardless of whether the bacterial host is replicating its own DNA, and every time a plasmid vector is replicated, so is the introduced DNA that it contains. ("https://www.sciencelearn.org.nz/resources/1900-bacterial-dna-the-role-of-plasmids").

They are key vectors of horizontal gene transfer and essential genetic engineering resources.

They were used in this research to express proteins from *A. thaliana*.

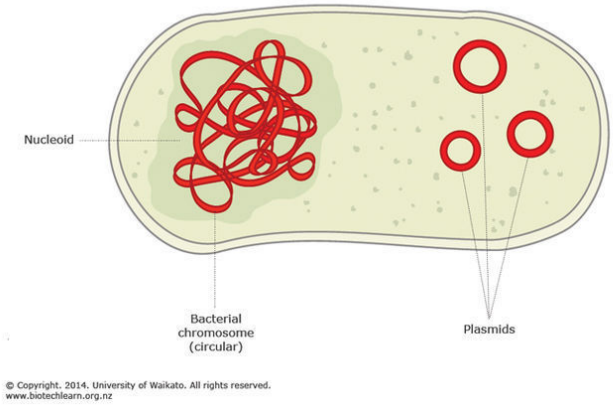


Figure 8. Example of host cell (University of Wakaito, 2014)

Two different kinds of mutant were used for the assays:

- ✓ ***E. coli* LB2003→** *E. coli* has three major K^+ uptake systems (Kdp, Trk and Kup) expressed in the inner membrane. The background K^+ influx activity in the triple K^+ transport-deficient *E. coli* strain is so low that the increased K^+ uptake permeability resulting from the exogenous gene products can be easily measured.
- ✓ ***E. coli* TO114→** *E. coli* has three major K^+ efflux systems (ChaA, NhaB and NhaA) expressed in the inner membrane. The efflux activity of *A. thaliana* genes can be easily measured in the *E.coli* TO114 mutant that has a lack of the three efflux systems.

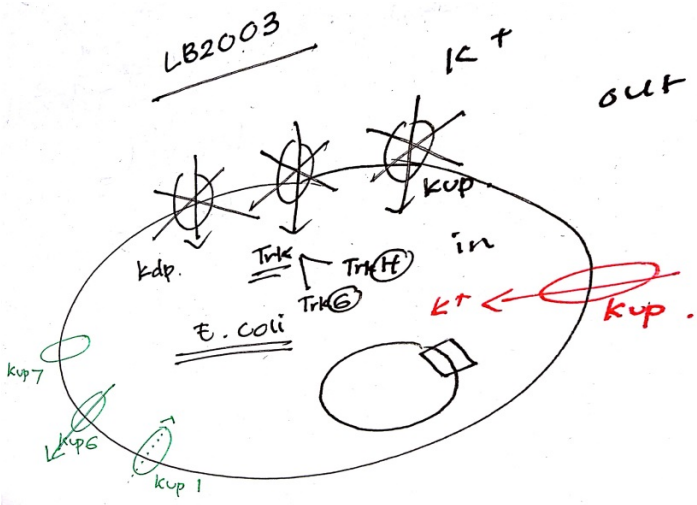


Figure 9. E. coli LB2003 (mutant)

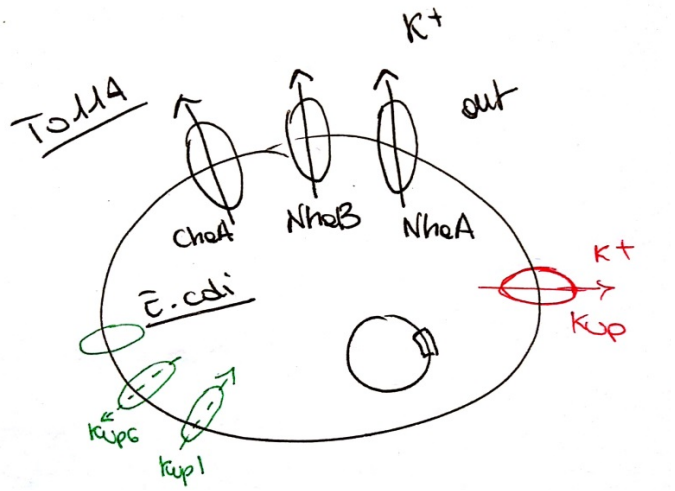


Figure 10. E. coli TO114 (mutant)

1.3 What is a plant cell?

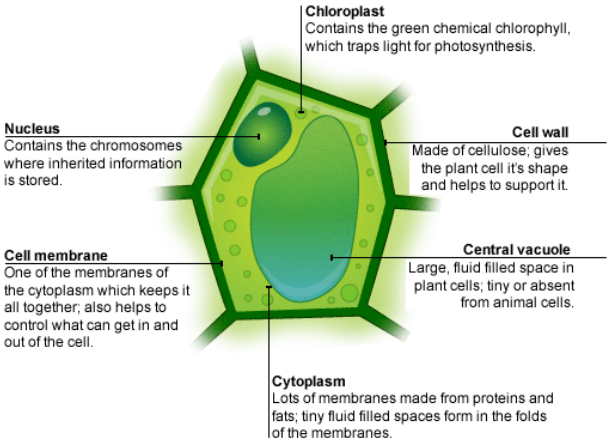


Figure 11. . Plant cell
(http://www.bbc.co.uk/staticarchive/9b7156a275ad2a777763855f15f5446dfeb57c90.gif)

Plant cells are eukaryotic cells indeed the DNA is enclosed within the nucleus. The most important difference between an animal and plant cell is the presence of the cell wall outside the cell membrane of the plant cell and the photosynthetic organelles.
The wall is constituted of cellulose and its main function is providing support and rigidity. There are many organelles that conduct specific functions necessary for survival and normal operation of the cells like producing hormones, enzymes and all metabolic activities of the cell. (https://biology.tutorvista.com/animal-and-plant-cells/plant-cell.html).
Like the bacteria plasma membrane, the cell membrane is the outer boundary of the cell. It's inside the cell wall and it's semi permeable, allowing only specific substances to pass through and blocking others.

1.3.1 Arabidopsis thaliana

Figure 12. A. thaliana growing in the laboratory

A.thaliana is a small plant in the mustard family that has become the model system of choice for research in plant biology ("Meinke, Cherry, Dean, Rounsley, Koornneef, 1998").
It has a small genome and its entire life cycle is completed in 6 weeks.
Everything about *A. thaliana* is small: flowers are 2 mm long, seeds are 0,5 mm in length at maturity and leaves are covered with small unicellular hairs.
The greatest properties are:

- Smaller and simple genome
- Convinience and abundance
- Genome size:

a. Nuclear: 125Mb
b. Plastid: 154Kb
c. Mitochondria: 365 Kb
d. Low amount of repetitive DNA

Plants can be grown in Petri plates or maintained in pots located either in a greenhouse or under fluorescent lights in the laboratory.
The genome is organized into five chromosomes and contains 20000 genes.
It has **homologous genes** with *E. coli*.
Two genes are homologous when their DNA sequence derives from a common origin and may or may not have the same function.
It's for this reason if *E. coli* is used as host cells on the study of *A. thaliana* membrane transporters: *At*KUP1 and *At*KUP6.

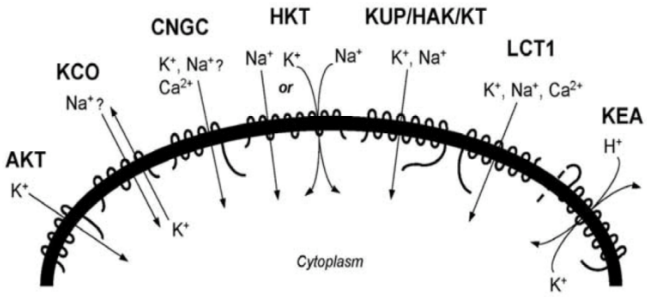


Figure 1. Plant K^+-permeable cation transporters. There are at least seven families of K^+-permeable transporters in plants that have been characterized to date (see text for details). The predicted structures of K^+ channels, CNGCs and HKT1 transporters are supported by experimental evidence (Doyle et al., 1998; Henn et al., 1995; Kato et al., 2001; Lopes et al., 2001; Sokolova et al., 2001; Uozumi et al., 1998). Structure prediction for KUP/HAK/KTs, LCT and KEAs was performed with the HMMTOP program (Tusnady and Simon, 1998); whether these proteins contain loop structures at their outer surface remains to be investigated.

Figure 13. From Molecular mechanisms of potassium and sodium uptake in plants- Pascal Mäser, Markus Gierth & Julian I. Schroeder

*At*KUP1 and *At*KUP6 are two transport systems belonging to one of the six major families of *Arabidopsis* cation transporters that are permeable to K^+:

1. Shaker- type K^+ channels;
2. 'two-pore' K^+ channels;
3. Cyclinucleotide-gated channels;
4. Putative K^+/H^+ antiporters;
5. *KUP/HAK/KT transporters;*

6. HKT transporters.

Arabidopsis has 13 KUP/HAK/KT genes. This variety may indicate functional redundancies; however, there is also evidence for functional diversification among individual KUP/HAK/KT genes.
The KUP/HAK/KT family comprises several candidates for root K^+ uptake transporters.
The concentration of potassium in living cells is very high and in plants, particularly, may reach up to 8 % of the dry weight. The high abundance of this cation in the cell implies its function in maintenance of cellular osmolality and compensation of negative electrical charges associated with organic molecules. At the biochemical level, it's essential for activity of various cytosolic enzymes.

1.4 Microbial growth curve

The growth of microbial cell is characterized by a curve. There are four different phases in the bacteria growth that can be classified in:

1. ***Lag Phase***: Immediately after inoculation of the cells into fresh medium, the population remains temporarily unchanged. Although there is no apparent cell division occurring, the cells may be growing in volume or mass, synthesizing enzymes, proteins, RNA, etc., and increasing in metabolic activity.
   The length of the lag phase is apparently dependent on a wide variety of factors including the size of the inoculum, time necessary to recover from physical damage or shock in the transfer, time required for synthesis of essential coenzymes or division factors and time required for synthesis of new enzymes that are necessary to metabolize the substrates present in the medium.

2. ***Log (or Exponential) Phase:*** The exponential phase of growth is a pattern of balanced growth where in all the cells are dividing regularly by binary fission and are growing by geometric progression. The cells divide at a constant rate depending upon the composition of the growth medium and the conditions of incubation. The rate of exponential growth of a bacterial culture is expressed as generation time, also the doubling time of the bacterial population.

3. ***Stationary Phase:*** Exponential growth cannot be continued forever in a batch culture (e.g. a closed system such as a test tube or flask). *Population growth is limited by one of three factors:*
   1. accumulation of toxic product and/ or exhaustion of available nutrients;
   2. accumulation of inhibitory metabolites or end products;
   3. exhaustion of space, in this case called a lack of "biological space".
      During the stationary phase, the number of new cell produce balance the number of that cell die resulting in steady state.

4. ***Death Phase:*** If incubation continues after the population reaches stationary phase, a death phase follows, in which the viable cell population declines.
   During the death phase, the number of viable cells decreases geometrically (exponentially), essentially the reverse of growth during the log phase.

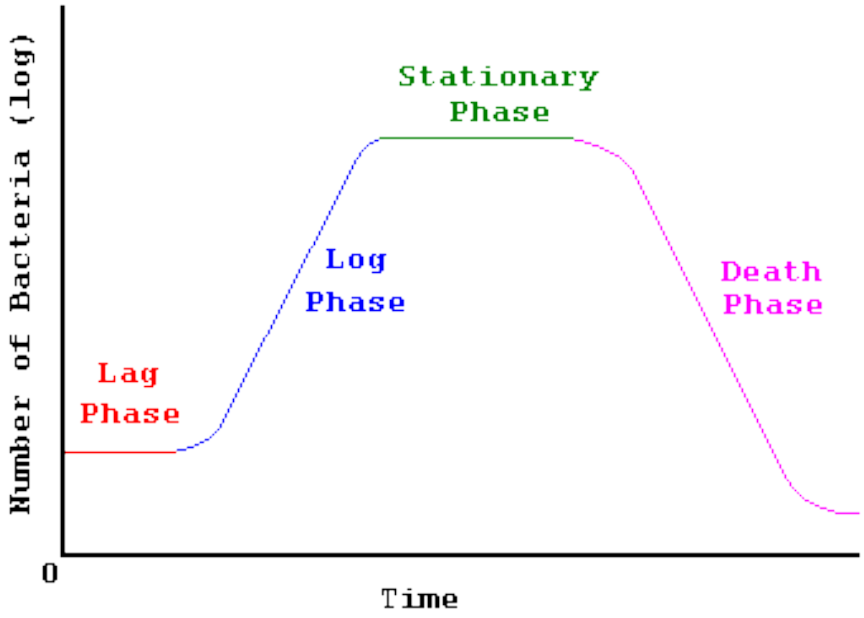


Figure 14. Curve of growth (https://www.peakprosperity.com/forum/population-growth-s-shaped-not-exponential/7250)

1.5 Potassium

Potassium (K^+) is an essential macronutrient for plant development and growth. It's the most abundant cation in plant cells. K^+ is taken up from a wide range of external concentrations $[K^+]_{ext}$ which can vary from 0,1 to 10 [mM].
The K^+ uptake can be affected by some environmental stresses as salinity, metal toxicity and drought but they can be dealt with the increase of K^+ supply.
Potassium has two important roles in the link with crop production and they are: K^+ reduces the effects of pests and disease on plants and K^+ is important in the onset of sodium (Na^+) toxicity.
K^+ plays major biochemical and biophysical roles in plants. It is demanded for maintenance of the photosynthetic apparatus and its deficiency reduces photosynthetic activity, chlorophyll content and translocation of fixed carbon.
For rapid uptake and transport of K^+ throughout the plant and between different cell compartments and cells within a tissue, membrane proteins are required to facilitate movement of K^+ through membranes. A wide variety of those transporters have already been identified at the molecular level, demonstrating the complex nature of K^+ transport.
These transports proteins include high-affinity transporters and ion channels encoded by a number of genes, resulting in a large range of functional, regulatory and tissue-specific properties. K^+ channels play a major role in the control of K^+ influx and K^+ efflux. Permeation rates through these channels are at least three orders of magnitude faster than those catalyzed

by pumps and carriers. K^+ channels are, in principle, similar to Ca^{2+} channels, their function is different. Potassium ions act directly as solutes, changing the osmotic potential in the compartments and thereby turgor, and, as carrier of charges, also the membrane potential.

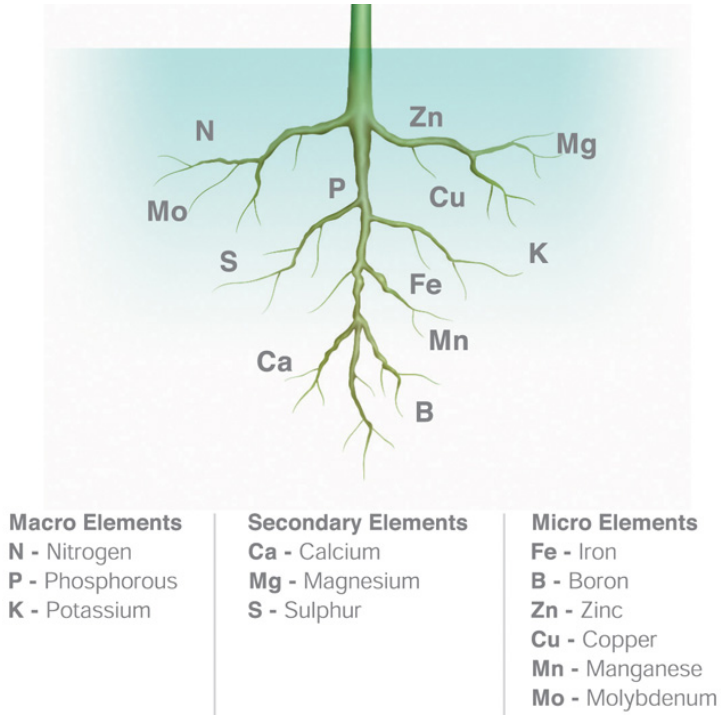


Figure 15. Important elements for plants (Petra Marschner, 1986)

1.6 Caesium

Caesium (Cs) is an alkali metal in group 1 of the periodic table and it's not an essential element for most living cells.

It has gained special world-wide attentions due to the release of considerably large amounts of radioactive caesium (^{134}Cs and ^{127}Cs) after the power plant accidents in Chernobyl in 1986 and Fukushima in 2011. This has led to studies of the environmental impact of Cs^+ contamination, decontamination attempts and bioremediation efforts as the research of microorganisms highly tolerant to Cs^+("Tanudjaja, Hoshi, Su, Hamamoto, Uozumi, 2017").

It is known the toxicity of Cs^+ to microorganisms, animal cells and plant cells.

Because of the similar chemical properties of Cs^+ and K^+, it has been proposed that Cs^+ can enter the cell via K^+ transport systems.

Cs^+ is frequently used as a K^+ channel blocker and most K^+ transport systems do not facilitate the uptake of Cs^+ instead of K^+.

Previous researches have reported that in *E. coli* only Kup, and not Trk or Kdp, can take up Cs^+. Kup participates in adaptation to high osmolality stress.

Kup transport system of *A. thaliana* is homolog to *Ec*Kup so due to the homology it has been proposed *At*Kup allows Cs^+ enters the cell via Kup.

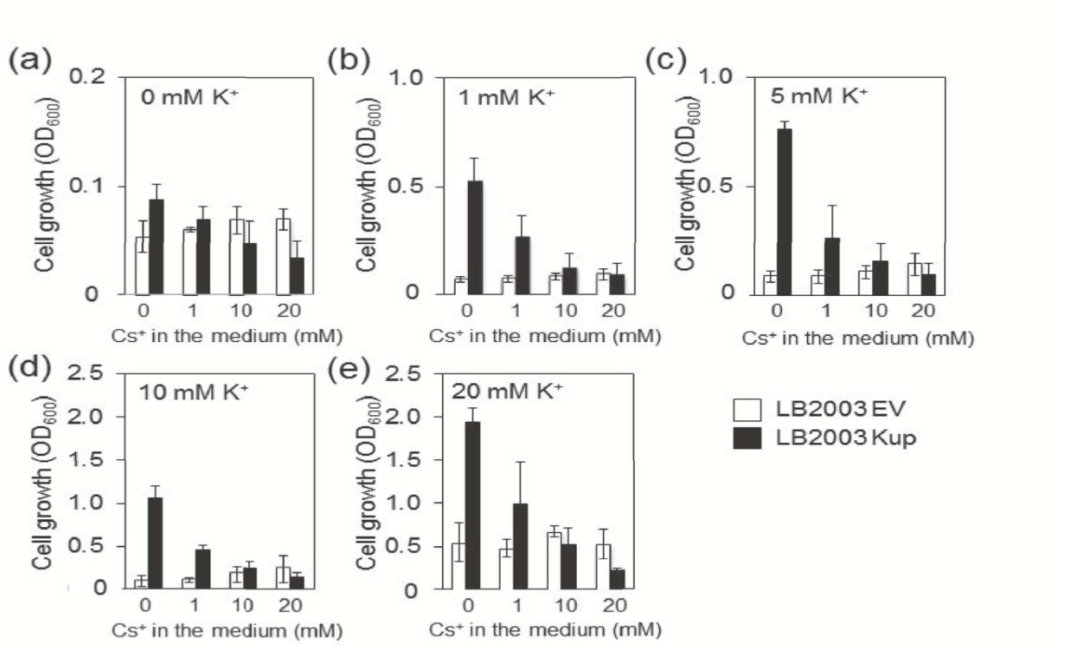


Figure 16. Comparison of growth of the K+ uptake system-deficient E. coli strain LB2003 transformed with Kup or EV at various concentrations of K+ and Cs+

Fig. 16 (*Ellen Tanudjaja, Naomi Hoshi, Yi-Hsin Su, Shin Hamamoto and Nobuyuki Uozumi, 2017*) shows the cell growth with different K^+ concentration and Cs^+ in the medium. Increasing the Cs^+ concentration the growth is inhibited due to its toxicity.

1.7 A brief description of DNA recombination technology

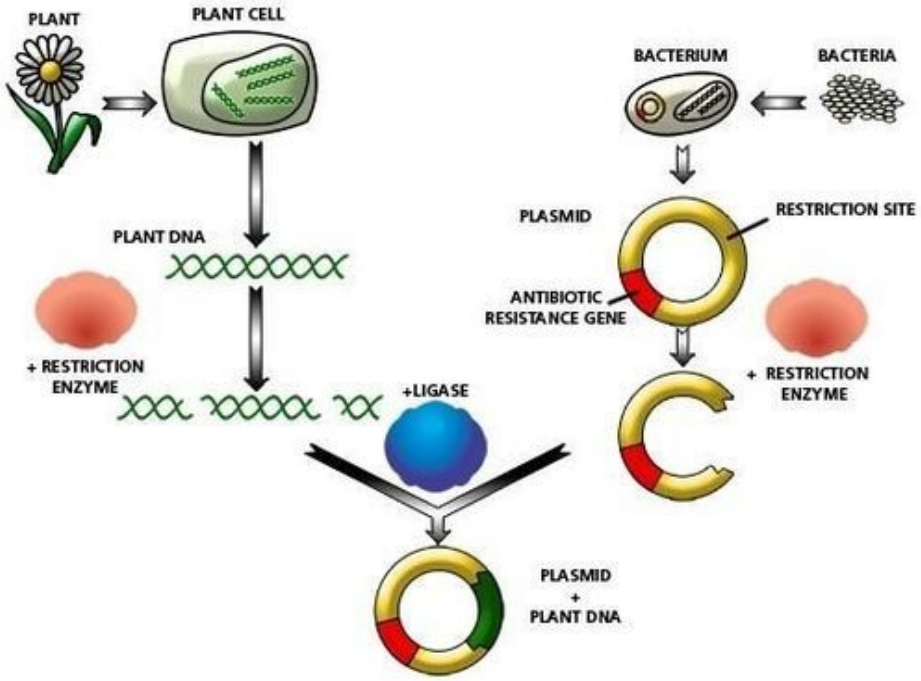


Figure 17. DNA cloning (University of Waikato, 2007)

In the laboratory Bacteria are commonly used as host cells for making copies of DNA because they are easy to grow.

They are incredibly versatile organisms that have unique ability to take in foreign DNA and copy it. This is a great advantage and helps them survive changes in their environment.

Plasmids may be also contained in bacteria and they are used as vectors to carry foreign DNA into a cell.

Enzymatic cleavage is applied to obtain different DNA fragments using restriction enzyme (endo-nucleases) for specific target sequence DNA sites followed by DNA ligase activity to join the fragments to fix the desired gene in vector.

The vector is then introduced into a host organism, which is grown to produce multiple copies of the incorporated DNA fragment in culture, and finally clones containing a relevant DNA fragment are selected and harvested. ("Suliman Khan *et al.*, 2016").

The choice of the host system is an important decision and it depends on the desired recombinant protein.

Popular bacteria for the host of protein production are: Gram positive (*Bacillus subtilis, Corynebacterium glutamicum*) and Gram negative (*Escherichia coli, Pseudomonas fluorescence*).

In this research *E. coli* was used as host cell because characters are well studied, techniques for gene manipulation and expression are well established and it has homologous genes with *A. thaliana*.

Although there are disadvantages as the presence of outer membrane, containing lipopolysaccharide (endotoxin that causes inflammation), cannot secrete the expressed protein to culture media (cell disruption should be needed).

2. Purpose of the thesis

In the Uozumi laboratory the researches are focused on molecular and cellular biological elucidation of membrane transport and signal transduction in higher plant cells, microorganism and animal cells using genetic engineering, electrophysiological measurement and cell free transcription, translation and translocation system.

The present research is based on the study of *At*KUP1 and *At*KUP6 transport activity using *E. coli* LB2003/TO114 as a host cell for protein expression system.

This transporters family was tested also for the K^+ efflux activity that is particularly important for the maintenance of K^+ homeostasis in K^+-deprived plants.

Even if the research was mainly focused on the K^+ uptake and efflux, the uptake activity was performed also for other ions as Cs^+, Li^+ and Rb^+.

Those ions belong at the same group of period table (I group) that mean they have the same charge. If a transporter is able to uptake K^+ there are good chances it can also uptake the other ions.

3. Materials and Methods

3.1 Materials

3.1.1 Plant Materials

All work was done on plants of species *Arabidopsis thaliana*. The assays were focused on *At*KUP1 and *At*KUP6. Those proteins have been previously isolated from *A. thaliana* in another laboratory and provided for the experiments.
Sequences homologous to the *At*KUP1 and *At*KUP6 coding region were identified from the expressed sequence tag (EST) and non-redundant (NR) databases by using BLAST.

3.1.2 Bacteria

Two different mutants of *E. coli* have been used for the experiments.
E. coli LB2003 for the uptake assay and *E. coli* TO114 for the efflux assay. They were used as host cells for the expression of *A. thaliana* homologous genes. LB2003 and TO114 have a lack of membrane transporters. LB2003 is lacking of Kup, KdpA, trkH, trkG whereas TO114 is lacking of ChaA, NhaB, NhaA.

3.1.3 Solid and Liquid Medium

LBK300 solid/liquid medium was used for the cultures. It's a potassium rich medium.
The composition for 200 mL of MQ water is:
2g of Hypolipepton; 1g of Yeast extract; 1,492g of KCl.
The pH was adjusted to 7,5 with KOH.
For the solid culture medium it has to be added 4g of agar into Erlenmeyer flask. After mixing everything and adjusting the pH, the solution is autoclaved. Finally ,100 µL of ampicillin solution (100 mg/mL- final volume to 50 µg/mL) is added.
The solution with agar has to be supplied into 20 mL/plate while the liquid medium was stocked in a 200 mL bottle. Both of them have to be stocked into the fridge at 4°C.

Figure 18. LBK300 solid and liquid medium

3.1.4 Glycerol Stock

To performed the assay, it was necessary to have a glycerol stock of mutant cells at -80°C.
First of all, *E. coli* (LB2003 or TO114) is inoculated to 2 mL of KLB liquid medium + ampicillin. The solution obtained is incubated at 30°C overnight.
When the time's up, 500 µL of each culture is supplied into 1,5 mL tube and 500 µL of 30% glycerol is added and mixed gently.
Finally, the tubes are carefully frozen with liquid N_2.
The stock samples for the uptake and the complementation test are: LB2003 + pPAB404, LB2003 + pPAB404_*At*Kup1, LB2003 + pPAB404_*At*Kup6.
The stock samples for the efflux test are: TO114 + pPAB404, TO114 + pPAB404_*At*Kup1, TO114 + pPAB404_*At*Kup6.

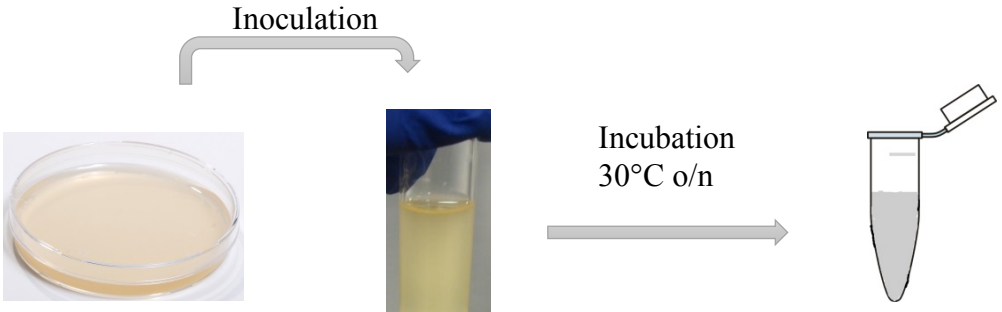


Figure 21. Culture

Figure 19. 2 mL KLB300+ Amp

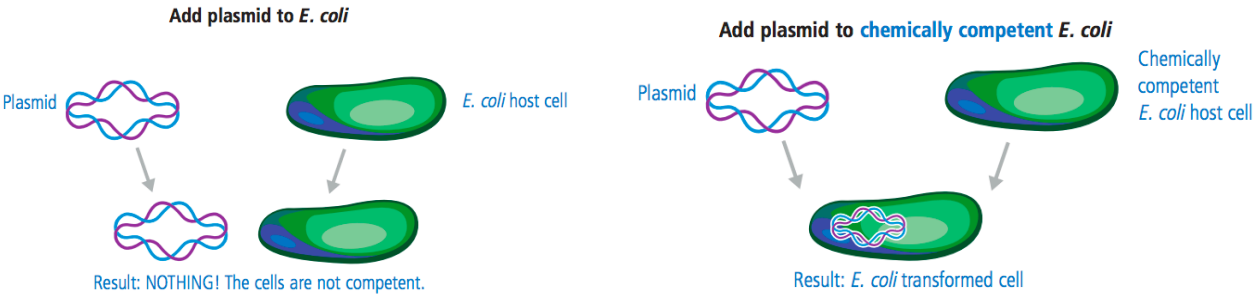
Figure 20. Sample stocks at -80°C

3.2 Methods

3.2.1 Transformation with heat shock

The first experiment was the transformation with heat shock. It's important to understand what a competent cell is. They are *E. coli* cells that have been specially treated to transform efficiently. There are two types of competent cells: chemically competent and electro-competent. If plasmid is simply added to *E. coli*, nothing happens. The cells must be competent.

Chemically competent cells were used in the experiment. They are treated with a buffer that contains $CaCl_2$ and other salts that disrupt the cell membrane creating "holes" that allow the plasmids to pass into the cell. Most researchers use chemically competent cells because they are less expensive, can be made in the lab and do not require special equipment.



The assay consists of:

a. Melting competent cells above ice
b. Take them into 1,5 mL tubes
c. Add 1-4 uL of plasmid solution
d. Put the tubes in ice for 10 minutes
e. Put them at 42°C for 60 seconds
f. Put them again in ice for 3 minutes
g. Take 150 uL into agar plate and spread with triangle comb
h. Incubate at 30°C overnight.

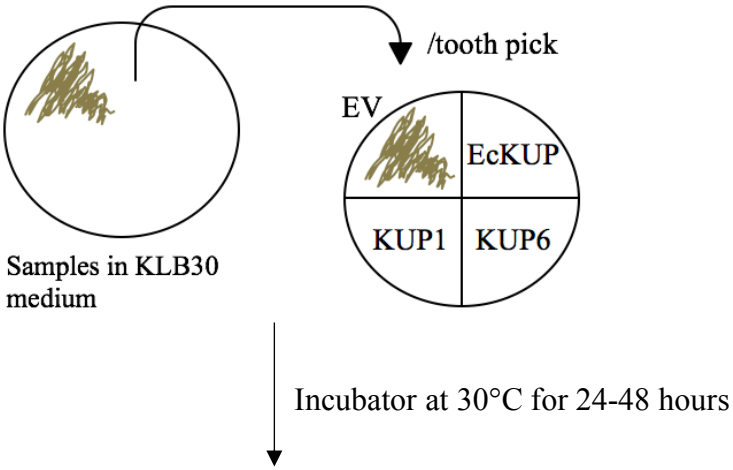
3.2.2 Complementation test in solid/liquid synthetic medium

→*Solid medium*

Samples:

1) LB2003 + pPAB404 (empty vector) = negative control
2) LB2003 + pPAB404_EcKup = positive control
3) LB2003 + pPAB404_AtKup1
4) LB2003 + pPAB404_AtKup6

Day 1: Preculture



Day 2:

After 24-48 hours the growth of the strains can be observed on the plate (KLB30 medium). The purpose of the experiment is to observe how the different K concentration changes the growth of every strains.

The next step is to inoculate EV, *Ec*Kup, KUP1 and KUP6 using a toothpick to K1, K5, K10 & K20. This step has to be done near the gas burner because of the possible contamination and after that all plates are incubated again at 30°C for 24-48 hours.

Day 3:

The results can be observed.

The experiment was repeated three times.

AtKUP1 and AtKUP6 were tested fort their ability to rescue LB2003 cells under potassium-limiting conditions.

Transformants were tested for the ability to grow in medium containing low potassium (~1/5 mM) and high potassium (~10/20 mM).

→ *Liquid medium*

The protocol for the assay with liquid synthetic medium instead of solid medium is almost the same.

Day 1:

The first step is preparing the pre-culture on KBL30 medium and incubating it at 30°C overnight. The strain is picked up with a toothpick from the KLB medium and it's transferred in the rich liquid medium (900 uL glucose stock, 100 uL mineral stock, 15 uL KCL 2M).

Day 2:

the next step is inoculated the pre-culture in media containing different K^+ concentration as it was done with the solid medium. The culture mediums have the same composition of pre-culture but with a concentration of: 1mM, 5mM, 10 mM and 20 mM of K^+.
Before inoculating the pre-culture is needed:

- ✓ Harvest the pre-culture;
- ✓ Wash the cell with K-free buffer;
- ✓ Inoculate to K1, K5, K10 & K20;
- ✓ Incubate at 30°C.

Day 3:

The OD600 has been measured after 8 hours and again after 24 hours.

3.2.3 Cation Uptake experiment

Solutions necessary for the experiments:

I.

Table 1. 10% KO stock solution

10% KO stock solution:		**Final Concentration**
$Na_2HPO4 \cdot 12H_2O$	32,94 g	46 mM
$NaH_2PO4 \cdot 2H_2O$	7,16 g	23 mM
$(NH_4)_2SO_4$	2,1 g	8 mM
MQ Water	200 mL in total	

II. 6 mM $FeSO_4 \cdot 7H_2O$
III. 1 M $MgSO_4$
IV. 2 M KCl
V. 120 mM Tris-HCl
VI. 20% Glucose

All of those solutions have to be prepared and autoclave separately. When we want to start the assay, we need to add the solutions to the medium. First, they will be added to the pre-culture and after 7 hr they will add to the main culture.
To perform the experiment the medium has to be made.
KCl Rich Medium:

Table 2. KCl rich medium composition

	Pre-incubation	**Incubation**
Total Volume	10 mL	200 mL
10% KO stock solution	1 mL	20 mL
MQ Water	8,5 mL	170 mL

The mediums have to be autoclave and after that all the solutions can be add:

Table 3. Solutions added to the medium

1 M $MgSO_4$	4 uL	80 uL
20% Glucose	500 uL	10 mL
6 mM $FeSO_4 \cdot 7H_2O$	10 uL	200 uL
2 M KCl	150 uL	3 mL
Thiamine	a little	a little
1 M IPTG	-	20 uL
Amp (100 mg/ mL)	3 uL	60 uL

The experiment starts in the morning making the pre-culture for all the strains. The pre-culture is made in a 50 mL Erlenmeyer flask by adding all the solutions according to the above table near the gas burned. The cells (LB2003_EV/*At*KUP1/*At*KUP6) are picked up from the plate (rich medium) with an iron stick and inoculated in the flask.
The pre-cultures were incubated at 30°C for around 7 hr.

After 7 hours, the OD578 has to be check with the spectrophotometer to know how many pre-culture is necessary to have the target OD in the main culture (OD578= 0,05).

It's better doing a dilution because the spectrophotometer gives optimal results on the range 0,2-0,4. If the solution is too concentrate the results may not be accurate.

Table 4. Example of absorbance values after 7 hours (d10x: 10uL of water + 90uL of cells suspensions)

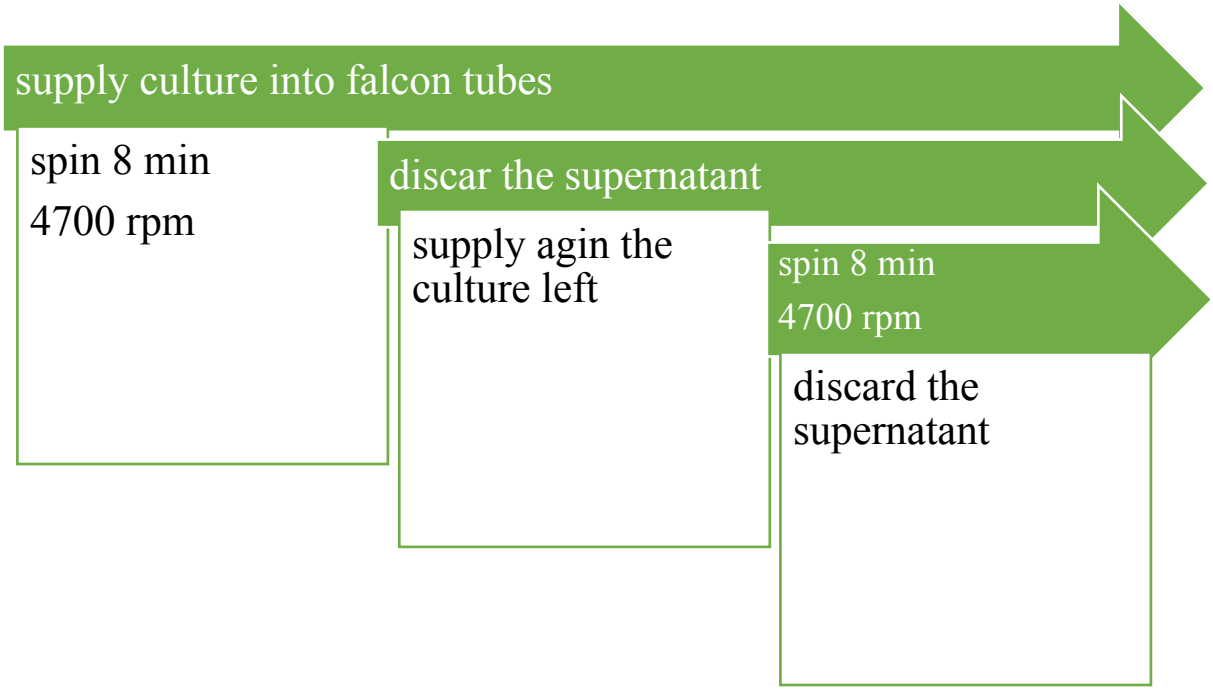
Samples	**Absorbance (578 nm)**
EV	0,29
KUP1	0,3
KUP6	0,33

We need to check and know the optical density because it provides an idea of the strains's growth.

It's important the strains are in the log phase when the experiment is performed.
Because of the different time of the growth the initial OD can be adjusted to reach the target value in the time we want.
First the experiment was performed using the same OD578=0,05 for each strain and then starting the assay at the same time for all of them.
But it was difficult to get clear results and to compare them so I decided to adjust the OD according to the growth of each strains. The assays were conducted at different time.

After 7 hours, the main culture can be prepared by mixing all the solutions in the 200mL flask medium and by adding the needed amount of pre-culture.
It has to be incubated at 30°C overnight.

The OD578 has to be checked to ensure the target that we need and if it isn't reached it has to be incubated again until the correct value will be reached.
When the main culture is ready the preparation of the cells can start.
The culture has to be transferred in centrifuge tube (falcon tube) and we have to spin in for 8 minutes at 4700 rpm.



The pellet has to be re-suspended in 6 mL of Buffer Tris HCl pH 8.0.
The absorbance shall be measured again but doing a 100 times dilution because in this case the cell suspensions are more concentrated:

d100x → 2 µL of cell suspensions and 198 µL of water

Table 5. example of value of 100 times dilution

Samples	**Absorbance (578 nm)**
EV	0,673
KUP1	0,718
KUP6	0,644

The quantity of cell suspensions has to be calculated to have a final volume and 10 mL and OD578=30.

Example of calculation:

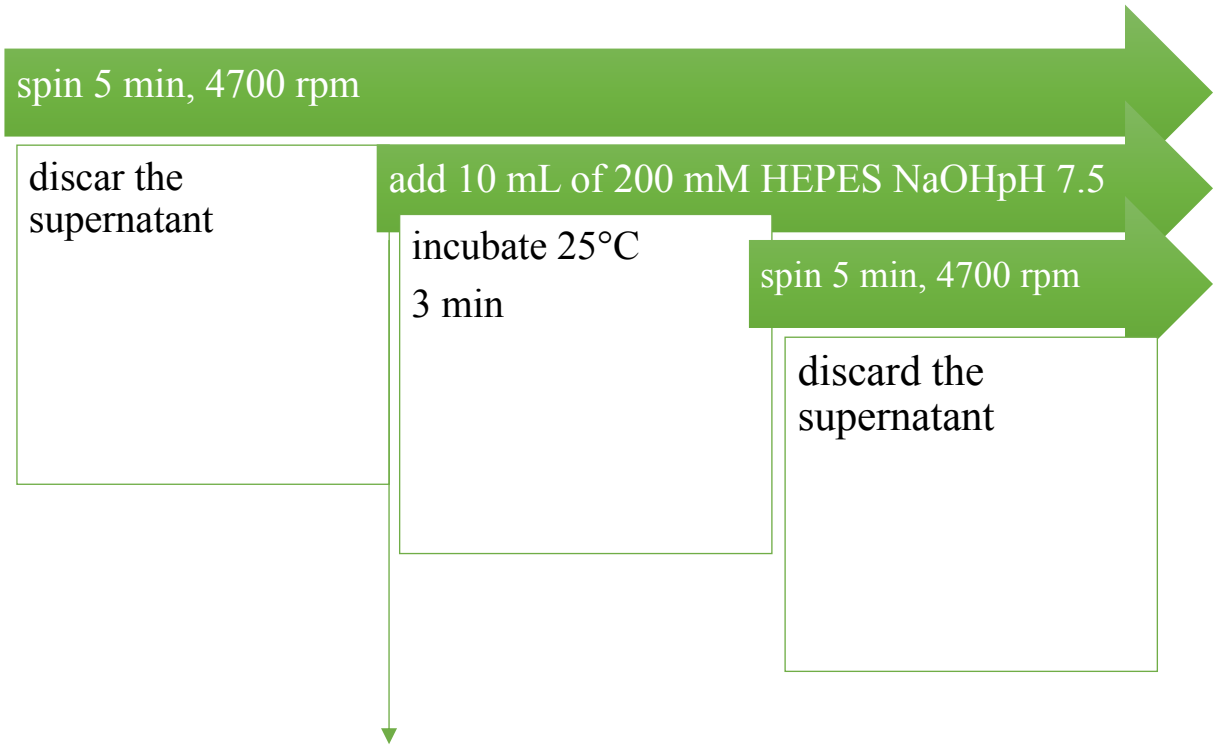
$$V_1 \cdot M_1 = V_2 \cdot M_2$$

$$\boldsymbol{EV}\colon\ V_1 \cdot 67{,}3 = 10 \cdot 30 \rightarrow\ V_1 = 4{,}45\ mL\ cell + 5{,}5\ mL\ 120\ mM\ Tris \cdot HCl\ (pH\ 8.0)$$

$$\boldsymbol{KUP1}\colon\ V_1 \cdot 71{,}8 = 10 \cdot 30 \rightarrow\ V_1 = 4{,}18\ mL\ cell + 5{,}82\ mL\ 120\ mM\ Tris \cdot HCl\ (pH\ 8.0)$$

$$\boldsymbol{KUP6}\colon V_1 \cdot 64{,}4 = 10 \cdot 30 \rightarrow V_1 = 4{,}66\ mL\ cell + 5{,}34\ mL\ 120\ mM\ Tris \cdot HCl\ (pH\ 8.0)$$

Then 100 µL of EDTA has to be added to each sample because EDTA is necessary to catch all the cations that aren't useless for the assay. The samples are incubated at 37°C for 30 minutes.
After the time necessary:



It's necessary to wash the pellet three times with the buffer 200 mM HEPES NaOH pH 7.5 to wash away the KCl medium.

When the wash phase is done, 10 mL of 200 mM HEPES NAOH pH 7.5 is added to the pellet that is mixed gently up and down by pipetting and eventually the OD578 is again calculated. Before starting the experiment it's necessary to incubate the tube again at 25°C for 20 minutes.
The check of the OD578 is it necessary to calculate how many buffer and cell suspensions are necessary to reach a final volume of 25 mL in the Erlenmeyer flask and OD578=3.
Example:

Table 6. Optical density values

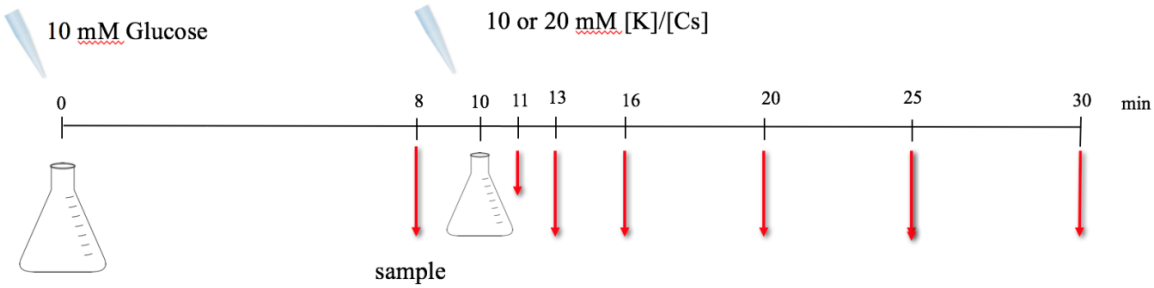
Samples	**Absorbance (578 nm) d100x**
EV	0,243
KUP1	0,166
KUP6	0,346

$$V_1 \cdot M_1 = V_2 \cdot M_2$$

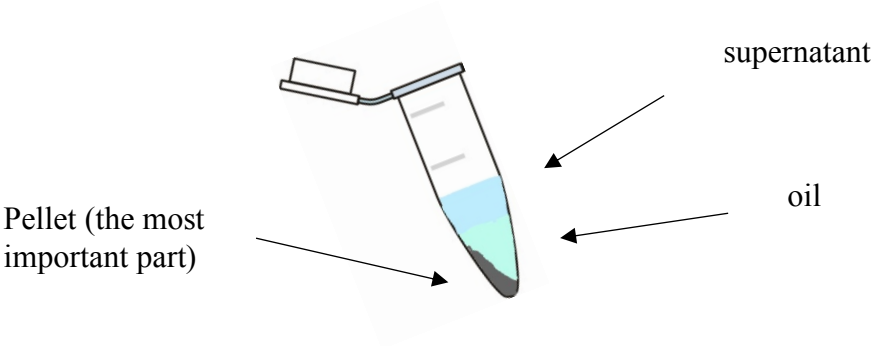
$\boldsymbol{EV}$: $V_1 \cdot 24{,}3 = 25 \cdot 3 \rightarrow V_1$
$= 3{,}08\ mL\ cell + 21{,}92\ mL\ 200\ mM$ HEPES NAOH (pH 7.5)
$\boldsymbol{KUP1}$: $V_1 \cdot 71{,}8 = 10 \cdot 30 \rightarrow V_1$
$= 4{,}52\ mL\ cell + 20{,}4\ mL\ 200\ mM$ HEPES NAOH (pH 7.5)
$\boldsymbol{KUP6}$: $V_1 \cdot 64{,}4 = 10 \cdot 30 \rightarrow V_1$
$= 2{,}17\ mL\ cell + 22{,}83\ mL\ 200\ mM$ HEPES NAOH (pH 7.5)

At this point, the main part of the assay can start. First of all, 150 µL of silicon oil has to be supplied to every Eppendorf tube (1,5 mL); seven tubes for every strain.
Six Erlenmeyer flasks are prepared, two for each strain, because the uptake of both Cs and K will be analyzed. They are placed in the shaker at 25°C.
The assay consists:

- start (0 min): add 600 µL 0,5 M glucose liquid;
- first sample (8 min): supply 1 mL of the cell suspension in the first tube and spin immediately at 15000 rpm for 1 minute;
- 10 min: supply 120 µL of 10 or 20 mM [K]/[Cs];
- take all the samples until 30 minutes.



After the spin, all tube will be divided into pellet, oil and supernatant:

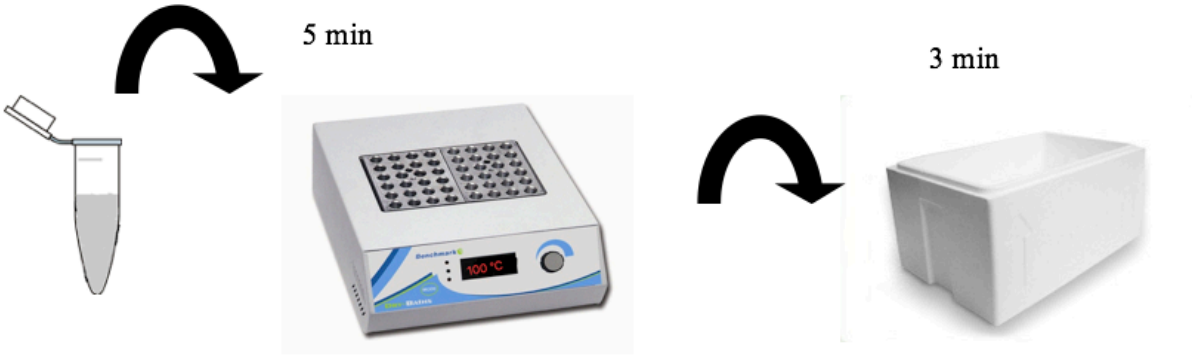


The important part to analyze the results is the pellet instead the supernatant and the oil have to be discarded. To do it an aspirator is used and so both the phases are sucked.
It's important to wash with MQ water the Eppendorf tube carefully during this operation in order to wash away residues of the supernatant.
It's important the water doesn't touch the pellet.

The steps to do are:

- suck supernatant;
- wash using MQ the inner wall of the tube, including the cap;
- suck again MQ and a part of the oil;
- wash again;
- suck MQ and all the oil.

The last step has to be done carefully because the pellet doesn't have to be suck and touch.
Now the samples are to be prepared to read the results. 500 µL of MQ water is supplied to each tube and after 15 minutes all of them are mixed with the vortex in order to re-suspend the pellet.
After that, 500 µL of 10% TCA (5 g of TCA in 50 mL of MQ water) are added and the solution is mixed well by inversion.
At this point it is obtained the death of *E. coli* cells using the heater.
First of all, the cap must be put in every tube because they contain water. Indeed, the water will boil with the high temperature in the heater and cause the opening of the sample if there isn't the cap.
The temperature in the heater is 100°C and the samples are left there for 5 minutes.
After this time, they are put on ice for 1 minutes.



Eventually all the samples are spinning in the centrifuge for 10 minutes so the pellet will be separate from the supernatant.
The supernatant (± 950 µL), that is important for analyzing the results of the assay, has to be transferred into new tube. If the results aren't read immediately it's possible to keep them in the refrigerator at 4°C until the ion density will be measured.
The pellet has to be keep in the refrigerator as well in order to measure the proteins with BCA protein assay.

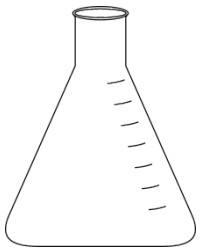
3.2.4 Potassium Efflux experiment

In order to understand totally the transporter mechanism of each strains, another type of assay was conducted. It concerns the analysis of K^+ efflux. The efflux is the passage from the inner part of the cell to the outer part of it.
In contrast to the uptake assay *E. coli* TO114 is used like host cell. It's used a different cell because of the different transformation efficiency.
Transformation efficiency is the efficiency by which cells can take up extracellular DNA and express encoded by it. *E. coli* TO114 has a lower transformation efficiency than *E. coli* LB2003.
First of all, the medium has to be made.
It's a rich medium and three flasks are necessary for the experiments:

Table 7. Medium composition

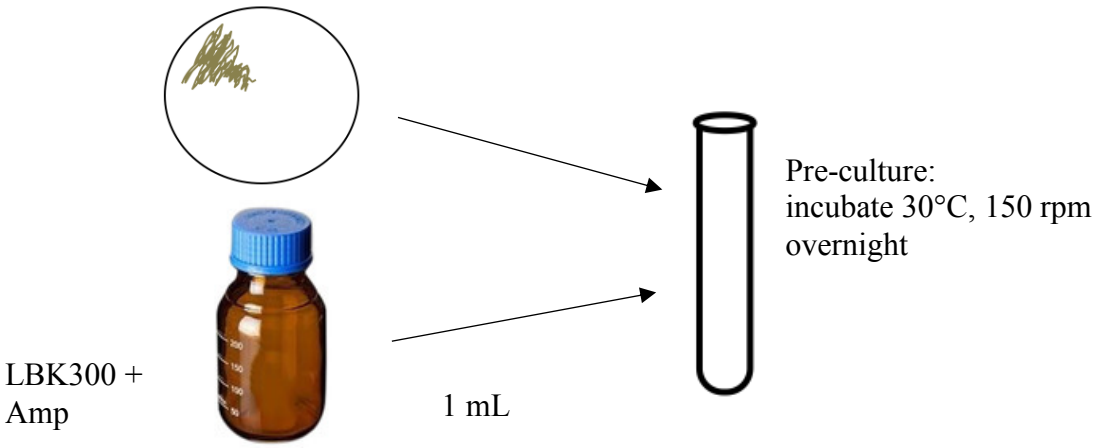
	Total Vol 120 mL
Hypolipepton	1,2 gr
Yeast extract	0,6 gr
KCl	2,6856 gr

Supply 40 mL x 3 flasks

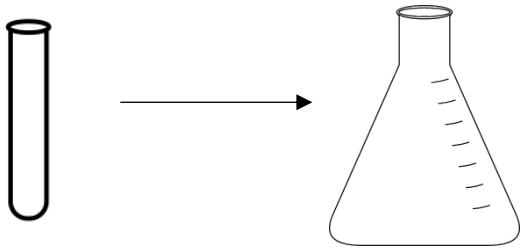


Erlenmeyer flask 100 mL
- Put Aluminum cover
- Autoclave

The first step to do is the pre-incubation of the cells into a liquid medium. The strains are taken with a tooth pick from the plates and they are inoculated in a test tube containing 1 mL of the liquid medium.
Pre- incubation:



The day after the main culture has to be made. 200 µL of pre-culture are added to the LBK300 medium together with 20 µL of Ampicillium and 100 µL of IPTG.
The flasks are incubated at 30°C and 150 rpm for around 7 hr until the OD660 will be 0,7-0,8.



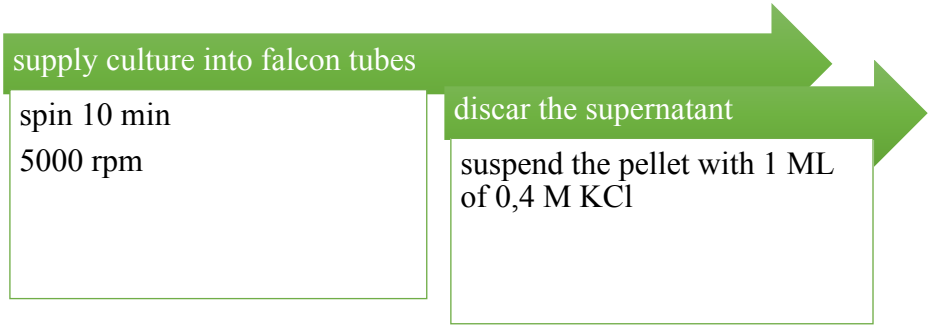
+ 200 µL pre-culture
+20 µL Amp (100 mg/mL)
+100 µL IPTG (100 mM)

After 7 hr, it's necessary to check the OD660:

Table 8. Example of values

Samples	**Absorbance (660 nm)**
EV	0,708
KUP1	0,636
KUP6	0,646

Table 2 is an example of adsorbance values that were measured. Every time the assay was repeated, the values were different but around the range 0,6-0,8.
When the necessary OD is reached, the assay can start.



The suspension has to be supplied into 1,5 mL tube and to spin again at 5000 rpm for 10 minutes. After that, the supernatant is discarded and 400 µL of 0,4 M KCl are added to re-suspend the pellet using the vortex.

Before starting the core of the experiment it's necessary to prepare the tubes. Like for the uptake experiment, also in this case 150 µL of silicone oil will be supply into 1,5 mL tube. It's function is to separate the buffer from the pellet. When the silicone step is done 200 µL of reaction buffer will be supply above the oil. After this step, it's important to spin all the tube because when the buffer is supplied it could happen the two substance are mixed. It's important to check that the meniscus faces downwards.

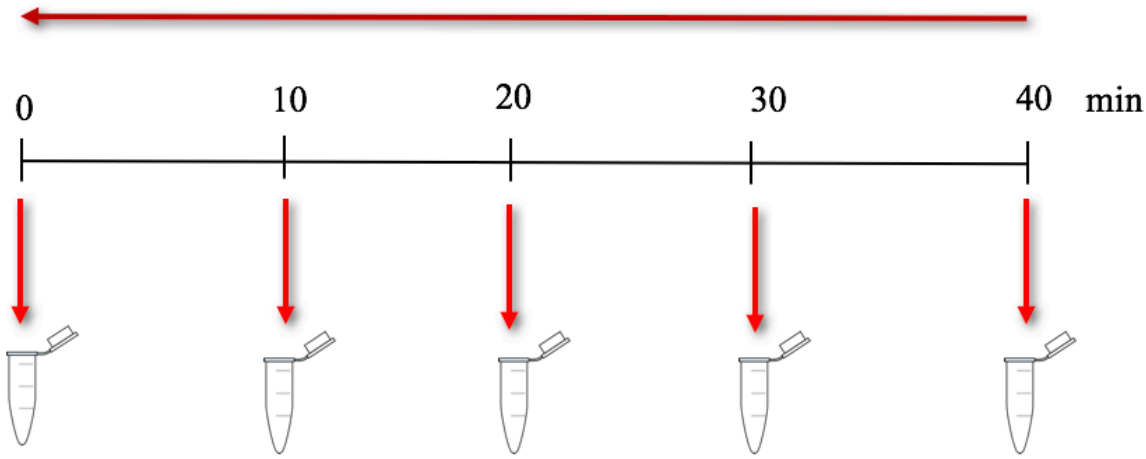
The assay was conducted in different conditions, i.e. different buffer with different pH.
First of all, it was performed with 20 mM HEPES NaOH pH 7.5 – 0,4M NaCl.

After that:

Table 9. Buffers that have been used

Buffer	pH
Buffer: Citric Acid – Na2HPO4	pH 5.0
MES Tris Ph 6,0 0,4 M NaCl	pH 6.0
HEPES NaOH Ph 7,0 0,4 M NaCl	pH 7.0
20 mM HEPES NaOH pH 7.5 0,4M NaCl.	pH 7.5
HEPES NaOH Ph 8,0 0,4 M NaCl	pH 8.0
CHES NaOH Ph 9,0 0,4 M NaCl	pH 9.0

When all the samples are filled with oil and buffer, the assay can start.
A small amount (16 µL) of the cell suspension has to be supplied inside the reaction buffer when the time comes.



When the time comes, the first fourth samples are spinning at 150 rpm for 1 minute. For the last sample (time 0), the suspension is supplied and immediately spun.
From this point, the steps to do are like the uptake experiment:

- discard the buffer by aspirant;
- wash the tube with MQ water two times;
- discard MQ water and the silicone oil;
- suspend the pellet with 500 µL of MQ water;
- wait for 15 minutes and suspend the pellet using the vortex;
- add 500 µL of 10% of TCA;
- put the cover on 1,5 mL tube
- put the tubes into the heater at 100°C for 5 minutes;

- put them into the ice for 3 minutes;
- spin all of them for 10 minutes at 15000 rpm;
- transfer ±950 µL of supernatant in new tubes;
- measure the ion concentration by AAS.

3.2.5 AAS Ion Measurement

Atomic Absorption Spectrometry (AAS) is a technique for measuring quantities of chemical elements present in samples by measuring the absorbed radiation by the chemical element of interest.

This is done by reading the spectra produced when the sample is excited by radiation. The atoms absorb ultraviolet or visible light and make transitions to higher energy levels. Atomic absorption methods measure the amount of energy in the form of photons of light that are absorbed by the sample. A detector measures the wavelengths of light transmitted by the sample, and compares them to the wavelengths which originally passed through the sample. A signal processor then integrates the changes in wavelength absorbed, which appear in the readout as peaks of energy absorption at discrete wavelengths. The energy required for an electron to leave an atom is known as ionization energy and is specific to each chemical element. When an electron moves from one energy level to another within the atom, a photon is emitted with energy E.

Atoms of an element emit a characteristic spectral line. Every atom has its own distinct pattern of wavelengths at which it will absorb energy, due to the unique configuration of electrons in its outer shell. This enables the qualitative analysis of a sample. The concentration is calculated based on the Beer-Lambert law. Absorbance is directly proportional to the concentration of the analyte absorbed for the existing set of conditions. The concentration is usually determined from a calibration curve, obtained using standards of known concentration.

First of all, it must to be calculated how much solvent (5% of TCA) it's needed.

For examples:

- 42 samples (14 samples for each strains) x 3,6 mL
- 10 K^+ standards x 10 mL
- 10 Cs^+ standards x 10 mL
- 2 Blank x 10 mL

Total: 371,2 mL → make 400 mL 5% TCA → 20 gr in 400 mL

To prepare the samples it's necessary to do a five times dilution

Falcone tube

After the

Total volume 4,5 mL:

- 3,6 mL %% TCA
- 0,9 mL sample

samples, the stock solution for K^+/Cs^+ standard is made.

stock solution $K^+/Cs^+ = \frac{10\ mg}{10\ mL}$ = 0,01 gr in 10 mL %% TCA

Standard + Blank (only 5% TCA)

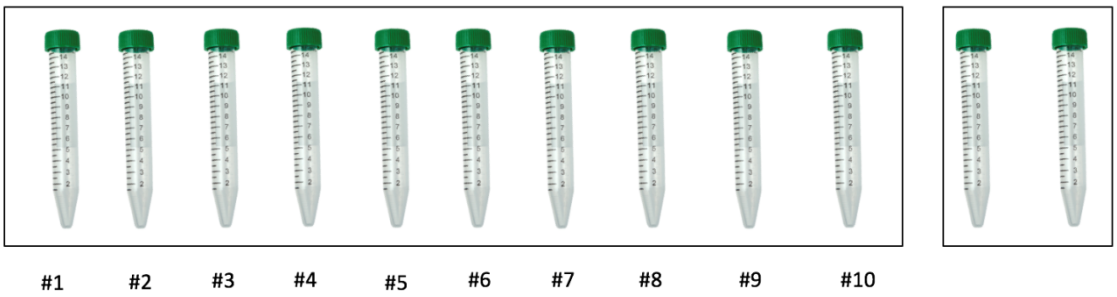


Table 10. Samples composition

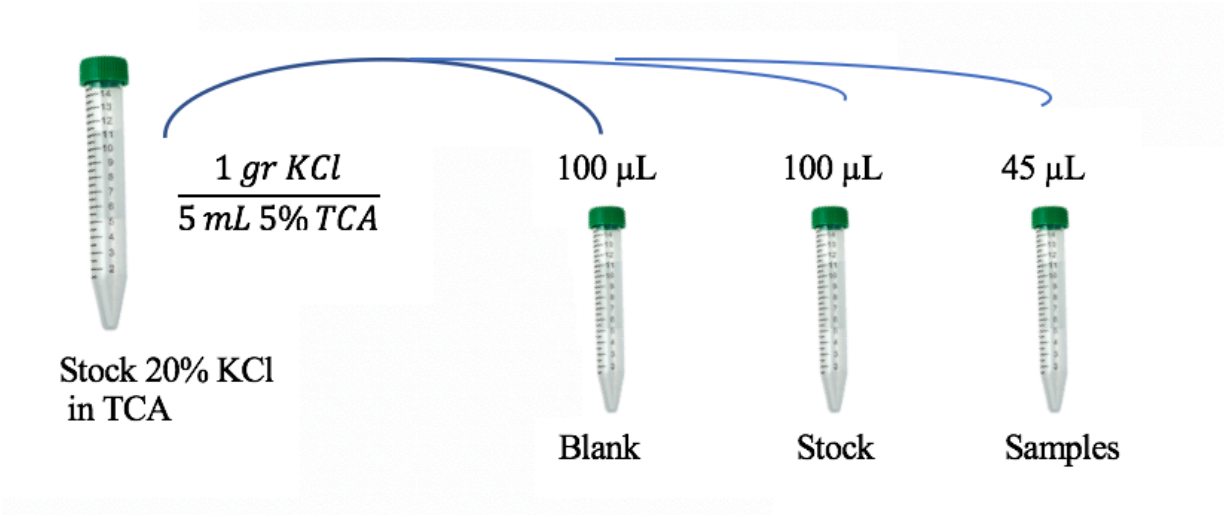
No of standards	**Final Concentration (mg/L)**	**Vol of stock solution**	**Vol of 5% TCA**
#1	0,5	5 µL	9 ml 995 µL
#2	1	10 µL	9 ml 990 µL
#3	2	20 µL	9 ml 980 µL
#4	3	30 µL	9 ml 970 µL
#5	4	40 µL	9 ml 960 µL
#6	5	50 µL	9 ml 950 µL
#7	8	80 µL	9 ml 920 µL
#8	10	100 µL	9 ml 900 µL
#9	15	150 µL	9 ml 850 µL
#10	20	200 µL	9 ml 800 µL

Number #9 and #10 are optional.

Only for Cs^+ measurements it has to be done one more step.

To read properly the results it's necessary to add K^+ in order of 1% of total volume:

$$+ \frac{20\ mg\ KCl}{10\ mL\ stock/blank/samples}$$



3.2.6 BCA protein assay

To normalize the results the total concentration of protein was measured using a biocinchoninic acid (BCA) assay. This assay has a reported dynamic range of 20-2000 µg/ml.

Figura 22. BCA protein assay kit

The working solution is made by adding a part of Reagent B and 100 parts of Reagent A:

$$A{:}B = 100{:}1$$

In each 1,5mL tube 500 µL of Elution buffer are added.

Table 11. Eluition Buffer composition

Elution Buffer	
1M NaOH	5 mL
MQ	20 mL
10% SDS	25 mL
Total	50 mL

After 15 minutes, the tubes are vortexed to suspend the pellets completely. To measure the protein, standards have to be made and supplied into 96 well plates and on it also the samples are supplied.
Standards:

Table 12. Standards composition

BSA (200 µg/mL)	**Elution buffer (µL)**	**SDW (µL)**
0	2	98
10	2	88
20	2	78
30	2	68

Before incubating at 37°C, 100 µL of working solution is added to each sample. After 1 hour, the absorbance of 565 nm is measured by multi plate reader using MQ water as blank.

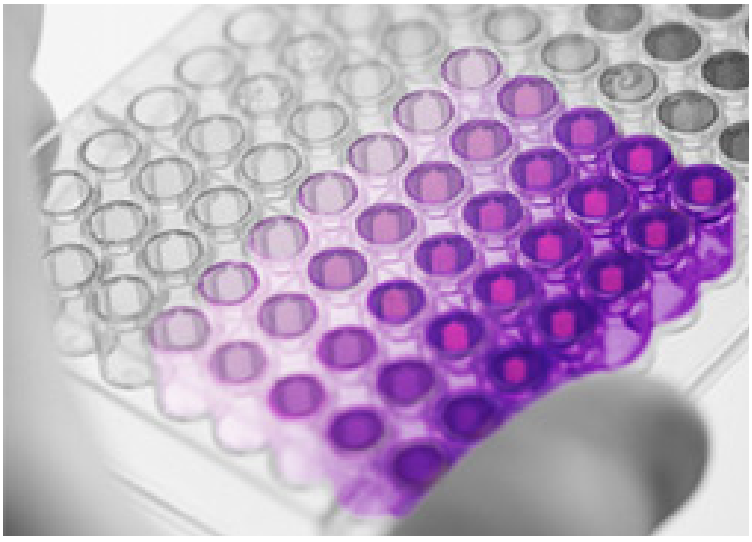


Figure 23. 96 well plates after 1 hr of incubation

4. Results

4.1 Transformation with heat shock

The experiment was performed with *E. coli* LB2003 and *E.coli* TO114 strains so probably the reason of the failure was low concentration of plasmid, low transformation efficiency or external condition that cannot be known or established. The low concentration of plasmid means the exogenous DNA is not expressed enough to see the growth in the Petri plate.
The transformation efficiency is defined as the number of transformants generated per µg of supercoiled plasmid DNA used in the transformation reaction. It can be calculated as following:

$$\frac{Number\ of\ Colonies\ on\ Plate\ (df)}{Amount\ of\ DNA\ plated\ (ng)} \times 1000\ ng/\mu g$$

The assay wasn't replicated but probably it could give positive results using a different *E. coli* strain.
For example, *E. coli* DHSα or *E coli* XL10-Gold that are known to have a higher concentration of plasmid.

4.2 Complementation test in solid/liquid synthetic medium

4.2.1 Solid medium

The assay was performed three times.
*At*KUP1 and *At*KUP6 were tested for their ability to rescue *E. coli* LB2003 cells under potassium-limiting conditions.
Remembering *At*KUP1 and *At*KUP6 are two transport systems belonging to one of the six major families of *Arabidopsis* cation transporters that are permeable to K^+. They've been expressed in *E. coli* LB2003 that is the host cell.
Transformants were tested for their ability to grow in medium containing low potassium (~1/5 mM) and high potassium concentrations (~10/20 mM).

- ***Ec*Kup** is *Escherichia coli* with all the potassium transport systems (Trk, Kdp and Kup). It is used as the positive control. We can see the behaviour of the mutants comparing it to the behaviour of the *Ec*Kup. As show in fig. 24 it has a positive growth with all the K^+ concentration.
- **EV** is an *E. coli* mutant lacking in all the transport systems. That's explain the different growth. As can be observed in fig. 24, it grew well only with high K^+ concentration (20 mM) indeed it wasn't able to grow in medium contain 1 mM of K^+.
- ***At*KUP1** is homologous to the Kup gene of *E. coli.* LB2003 cells containing a plasmid expressing *At*KUP1 were able to grow in medium containing ~20-10 and 5 mM of potassium but not 1 mM. This is a demonstration of functional complementation of an *E. coli* mutant with a plant transporter
- ***At*KUP6**, like *At*KUP1, is homologous to the Kup gene of *E. coli.* But the ability of this strain to rescue LB2003 cells under potassium limiting conditions is lower

compared to *At*KUP1 even if the growth is enough to be observed with 20 and 10 mM of potassium.

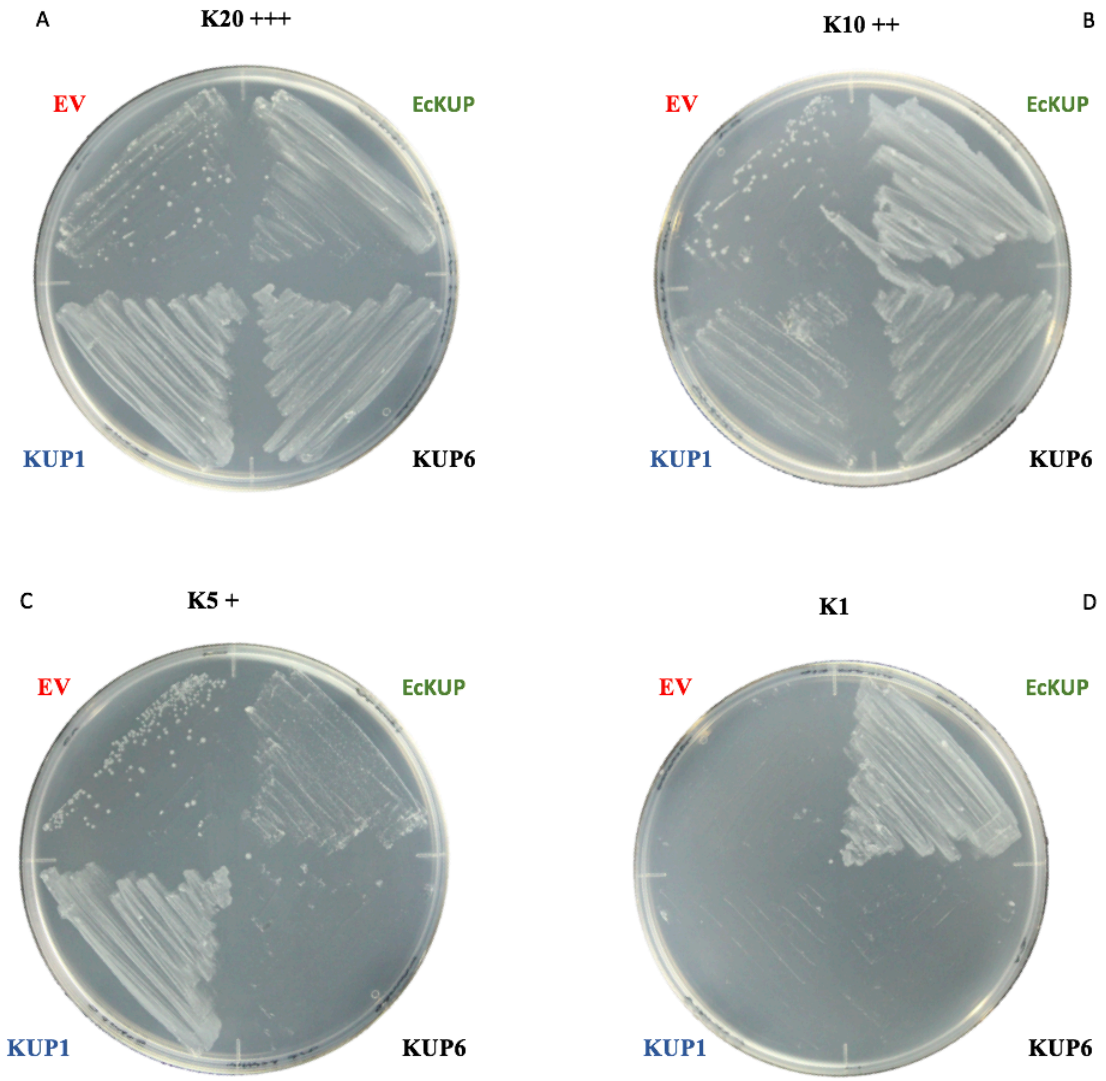


Figure 24. Results of first assay [K20++(20 mM K^+), K10++ (10 mM K^+), K5+ (5mM K^+), K1 (1 mM K^+), EV (empty vector), EcKUP (E. coli with all K^+ transporters), KUP1 (A. thaliana KUP1) and KUP6 (A. thaliana KUP6)]

The experiment was replicated a second time after a month using the same strain and transporters.

Fig. 25 shows *Ec*Kup has a positive growth with all the K^+ concentration as the first test. EV, as before, grew well only with high K^+ concentration (20 mM) indeed it wasn't able to grow in medium contain 1 mM of K^+. The growth of *At*KUP1 and *At*KUP6 is the same of the first experiment.

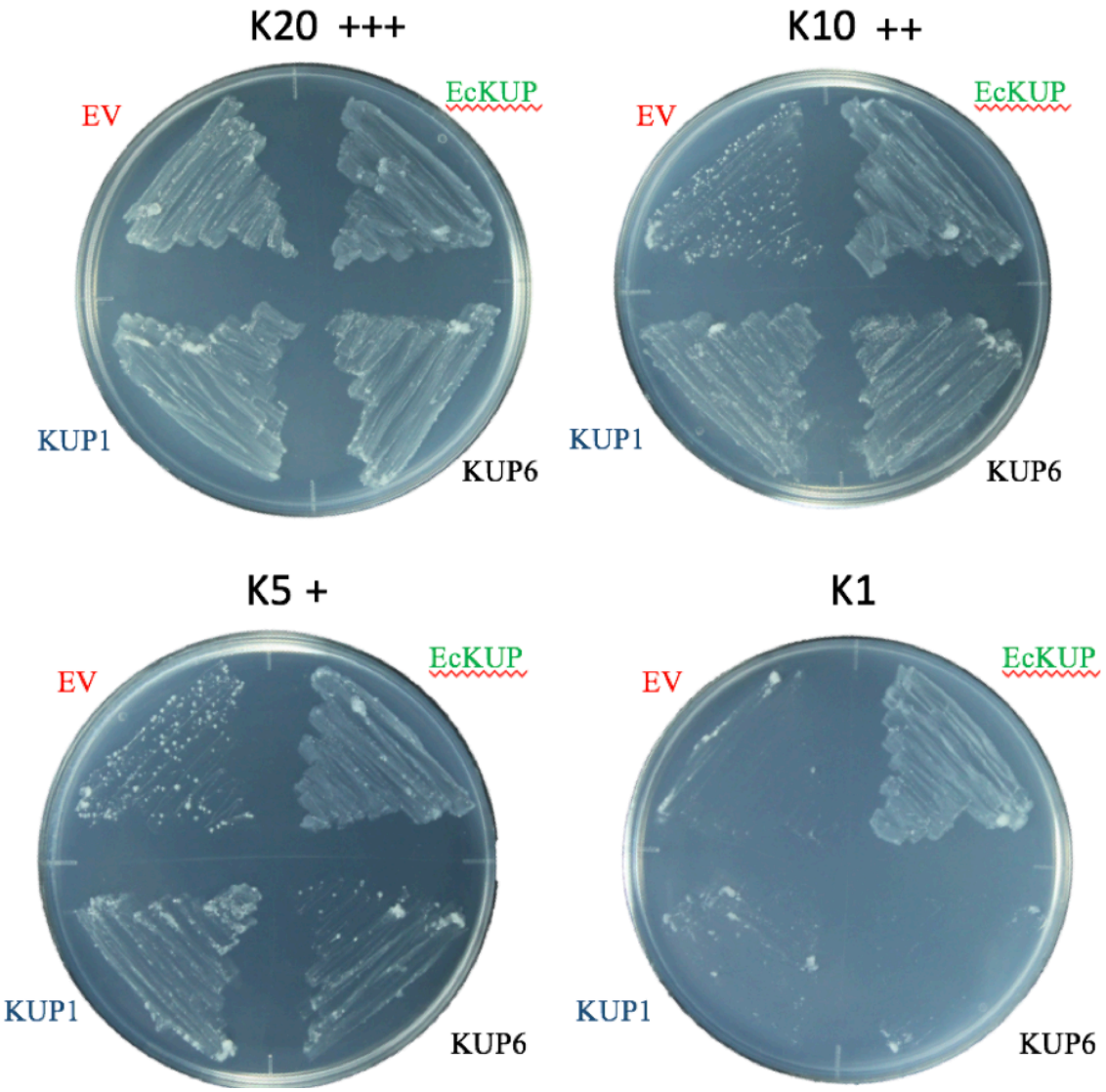


Figure 25. Results of second assay [K20++(20 mM K^+), K10++ (10 mM K^+), K5+ (5mM K^+), K1 (1 mM K^+), EV (empty vector), EcKUP (E. coli with all K^+ transporters), KUP1 (A. thaliana KUP1) and KUP6 (A. thaliana KUP6)]

Looking at the results of the third and last replication it can see differences in the growth of *At*KUP6.

While the other strains showed almost the same behaviour every time, *At*KUP6 showed a higher growth to all the K^+ concentration.

Fig. 26 shows that there is a low growth also at K1 while at this concentration there wasn't growth for this strain at all in previous replications.

Nevertheless, the cells stock that was used to perform the replication was the same, the results show differences.

These differences can be associated to the impossibility of recreating the same biological condition every time the assay is performed and sometimes it is affected by factor that cannot be controlled or manipulated.

That can justify different results.

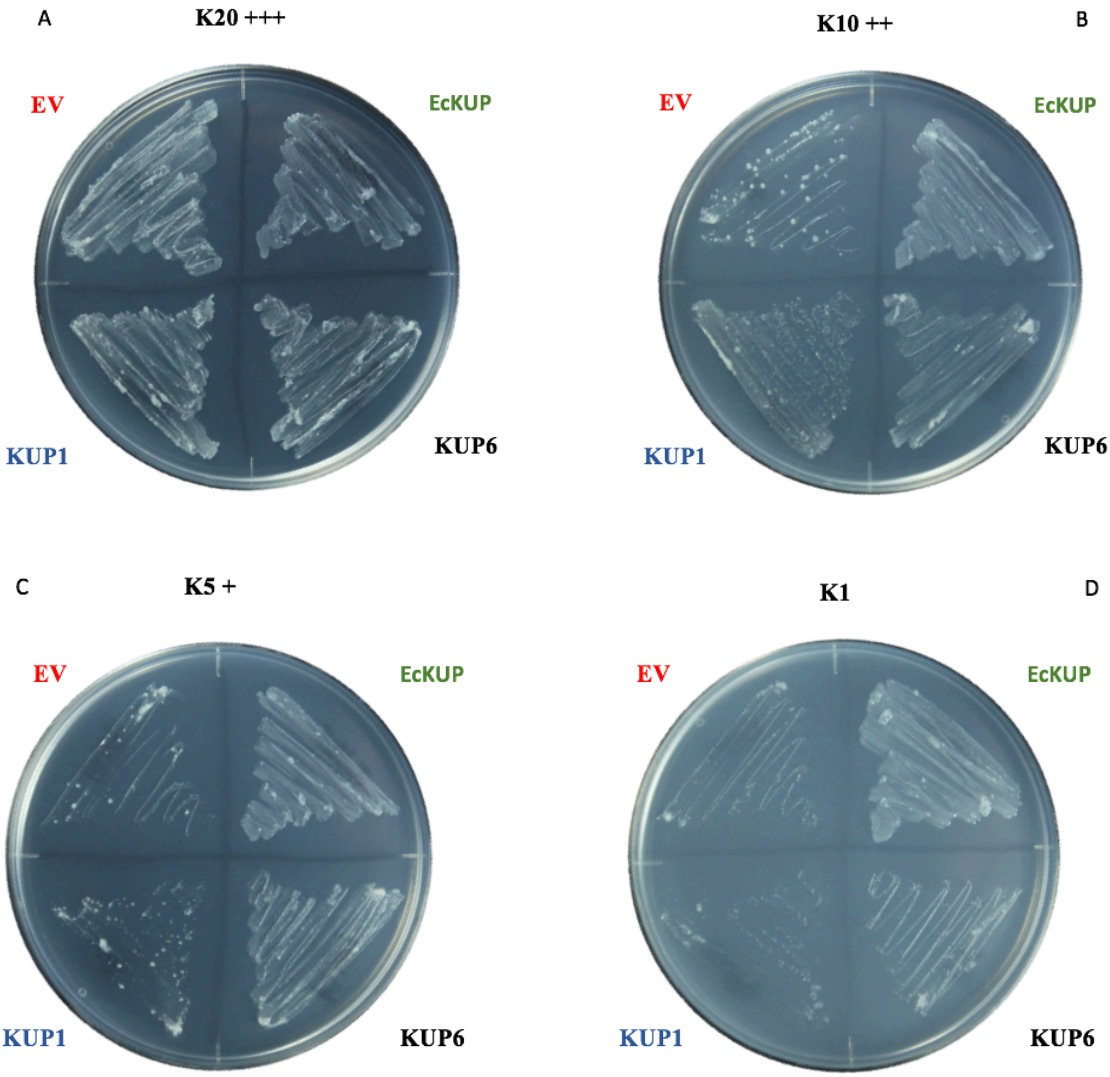


Figure 26. Results of first assay [K20++(20 mM K^+), K10++ (10 mM K^+), K5+ (5mM K^+), K1 (1 mM K^+), EV (empty vector), EcKUP (E. coli with all K^+ transporters), KUP1 (A. thaliana KUP1) and KUP6 (A. thaliana KUP6)]

4.2.2 Liquid medium

While a quality complementation ability can be observed with the solid medium, a quantitative ability can be analyzed with liquid medium.

It was observed that with ambient K^+ level of 1 mM, 5 mM and 10 mM K^+ strain *E. coli* LB2003 transformed with empty vector (EV) has a slow growth rate. Those results are compared with the results obtained for *E. coli* LB2003 transformed with KUP1 and KUP6.

As show in table 13, also for *E. coli* LB2003 transformed with plant K^+ channel the growth rate in slow with 1 and 5 mM K^+ instead looking at 10 and 20 mM the growth rate increases.

According to the previous growth test on solid medium, *At*KUP1 shows an increase already on the K10 medium while the growth rate of *At*KUP6 is still slow. At the end, both of them has an increased growth of *E. coli* LB2003 on the K20 medium.

Table 13. Optical density measured at 600 nm (OD600)

	1 mM KCl	***5 mM KCl***	***10 mM KCl***	***20 mM KCl***
EV	0,089	0,091	0,077	0,310
KUP1	0,087	0,181	1,696	3,183
KUP6	0,098	0,079	0,140	2,647

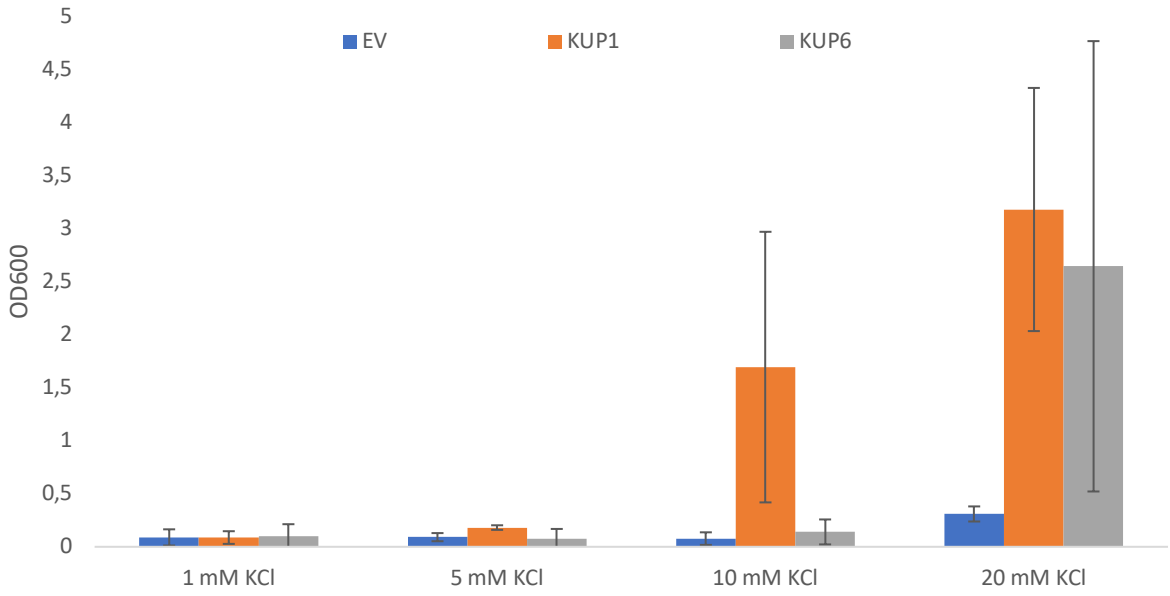


Figure 27. Growth test performed by adding k^+

Fig 27 shows the results of the first assay. How it can see, the error bar in some cases is really large. That means the results, that have been obtained in every replication, are really different. The reasons can be various as contaminations, mistakes during the test, different condition (as different T, different K^+ concentration). To gain results in higher detection (smaller error bar) the assay has to be replicated several times.

The assay was repeated by adding only Cs^+ instead of K^+ but the growth was probably inhibited and it's slow for each strain.

Cs^+ is a similar alkali metal element and it acts as a substitute for K^+ during the growth. The different is that Cs^+ is not an essential element for most living cells and it's toxic in microorganism, animal cells and plant cells.
Because there is no growth with only Cs^+, another test was performed by combing K^+ and Cs^+.
0,1 mM of K^+ was added in each medium with different concentration of Cs^+ (0/0,5/5/25 and 100 mM of Cs).
As show is fig. 28, the growth is still inhibited for each strain except for the positive control. *Ec*Kup shows an increase with low Cs^+ concentration but then increasing the Cs^+ concentration the growth suddenly decreases.

Table 14. Optical density measured at 600 nm (OD600)

	0mM Cs	***0,5 mM Cs***	***5 mM Cs***	***25 mM Cs***	100 mM Cs
EV	0,057	0,065	0,04	0,047	0,028
EcKup	0,7375	1,107	0,1845	0,099	0,006
KUP1	0,067	0,0605	0,0655	0,0575	0,0325
KUP6	0,054	0,7015	0,0565	0,059	0,033

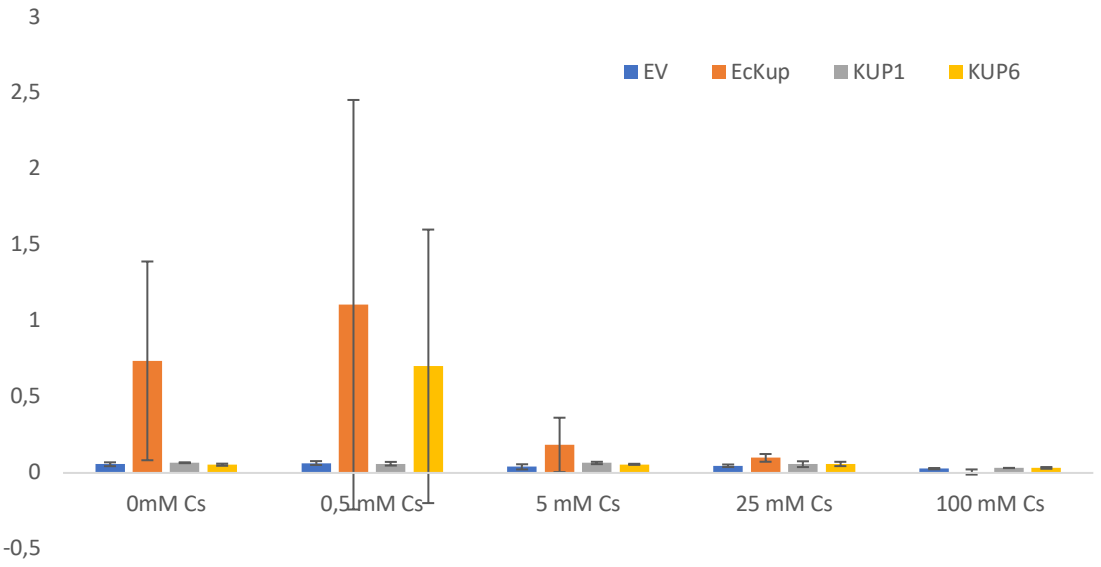


Figure 28. Growth test by combining K^+ and Cs^+

How it can see in fig. 28 the error bar in some cases in really large. The reasons are probably the same as before.

4.3 Cation Uptake experiment

By analyzing the growth, we found out that *E. coli* expressing *At*KUP1 is the faster cell while *E. coli* expressing EV or *At*KUP6 is slower and in particular it was more difficult to perform the assay for *At*KUP6 because of the growth time's fluctuations.
The OD578 used for the cation assay was:

- 0D578=0,05 → EV
- 0D578=0,03→*At*KUP1
- 0D578=0,04→ *At*KUP6

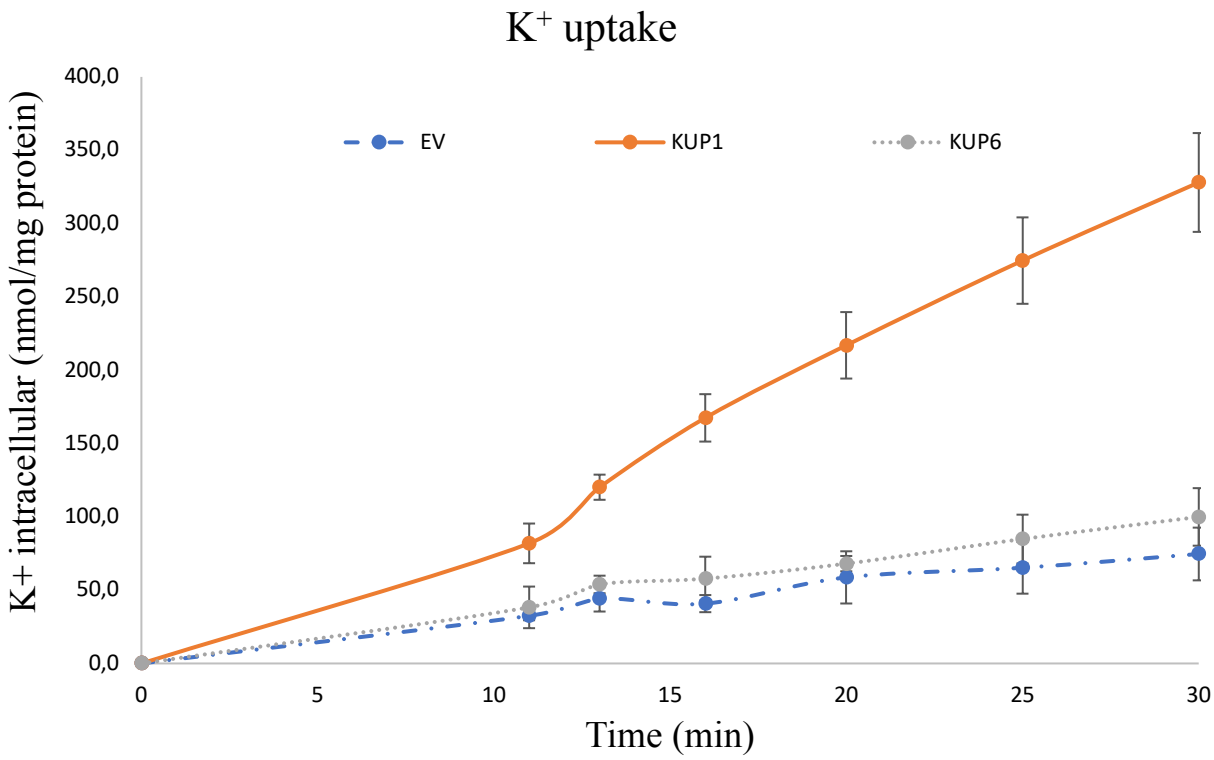


Figure 29. K^+ uptake activity

The diagram above shows the results of the assays. Each diagram relates at the average values and it shows also the error bar. Every assay was performed three times to get the average value and make sure of the accuracy of the data collected.
Three lines refer to EV, KUP1 and KUP6.
The complementation test shows a high ability of *At*KUP1 to rescue *E. coli* LB2003, indeed a lower activity of *At*KUP6. The same results should be showed with the uptake experiment.
As show in fig. 29, *E. coli* LB2003 expressing *At*KUP1 show enhanced uptake in contrast to cells transformed with empty vector as controls.
The uptake of potassium is constant until 120 µL of K^+. Then it suddenly increases until the end of assay is reached.
Compared *At*KUP1 and *At*KUP6 it can be easily observed the ability of KUP6 is almost the same of EV. That means it doesn't show a strong uptake activity and alone is not able to ensure the appropriate amount of K^+ required for the cells growth.
The uptake assay was performed also with other ions (Cs^+, Rb^+, Li^+) to investigate if the same protein is able to allow the influx of different ions or if it is selective to only K^+.

The results are showed in figure 30, 31 and 32.
Positive data was obtained for Cs^+(figure 30). Due to the similar chemical properties of Cs^+ and K^+, the results shows that Cs^+ enters the cells via K^+ transport systems.
According to the diagram, KUP1 is able to uptake both Cs^+ and K^+ so it is a high affinity transporter for these ions. While the ability of KUP6 to influx Cs^+ is not high indeed it is as EV activity.

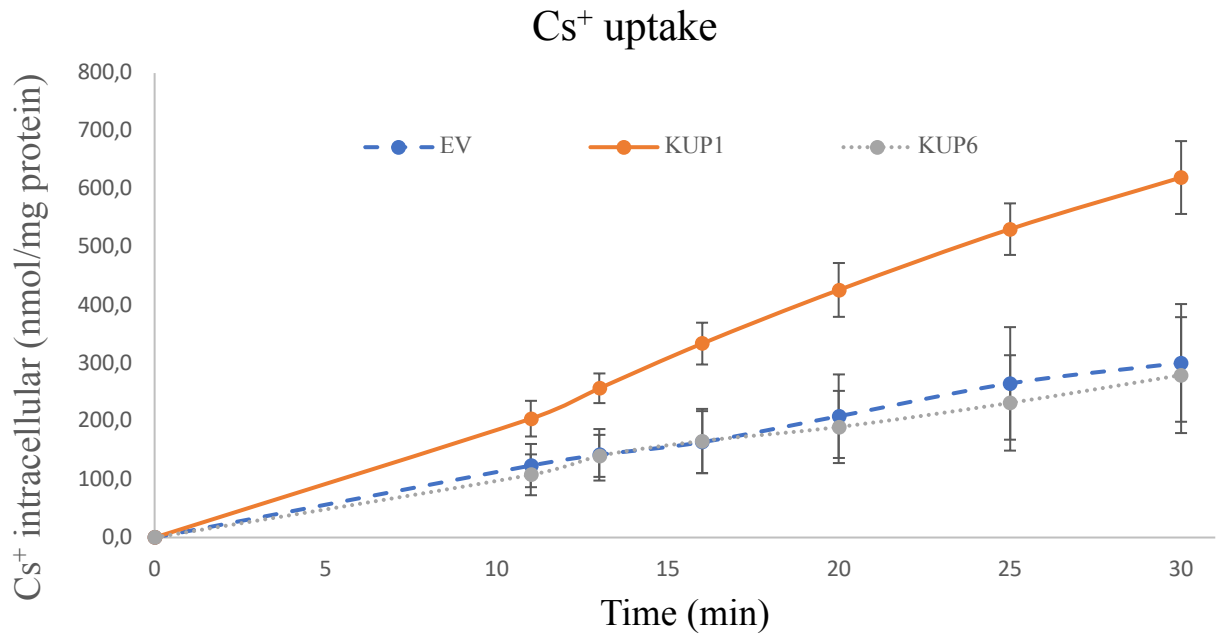


Figure 30. Cs+ uptake activity

The last experiments were focused on the analysis of Li^+ and Rb^+. As Cs^+, KUP1 shows a good uptake activity but the same cannot be affirmed for KUP6.
In conclusion, can be affirmed the uptake ability of *At*KUP1 is high and this protein is no selective to K^+ indeed thank to the other assay it shows it can uptake also different ions (Cs^+, Li^+ and Rb^+) that belong at the same group.
On the other hand, the uptake ability of *At*KUP6 is very low and it doesn't show positive data for other ions.

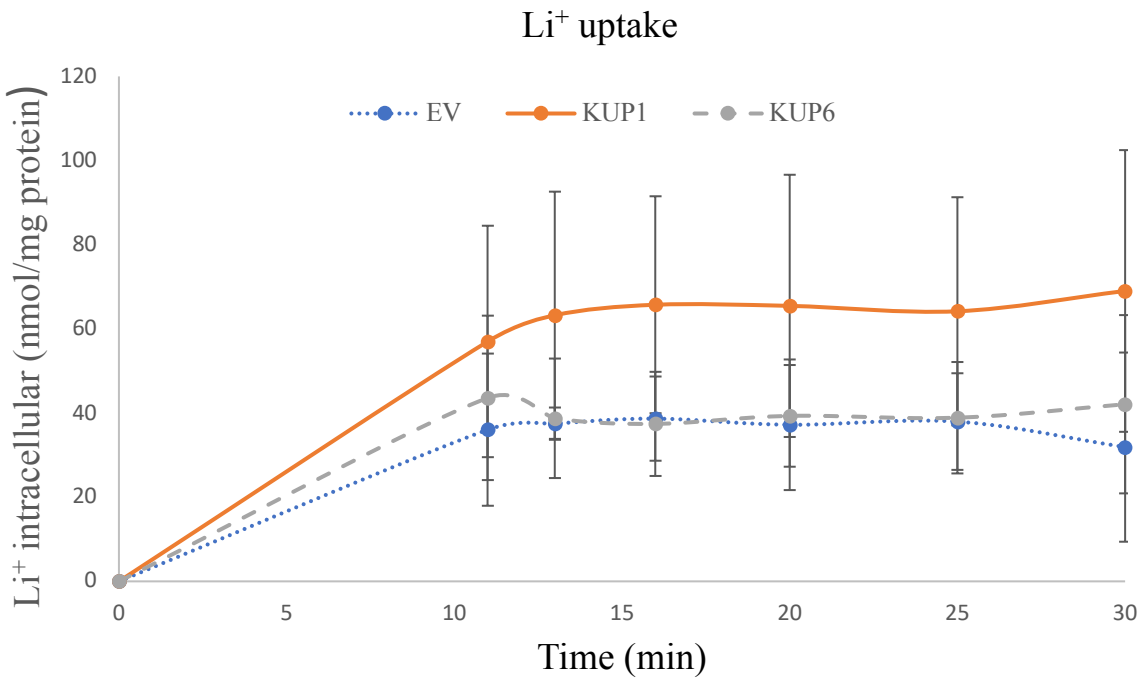


Figure 31. Li+ uptake activity

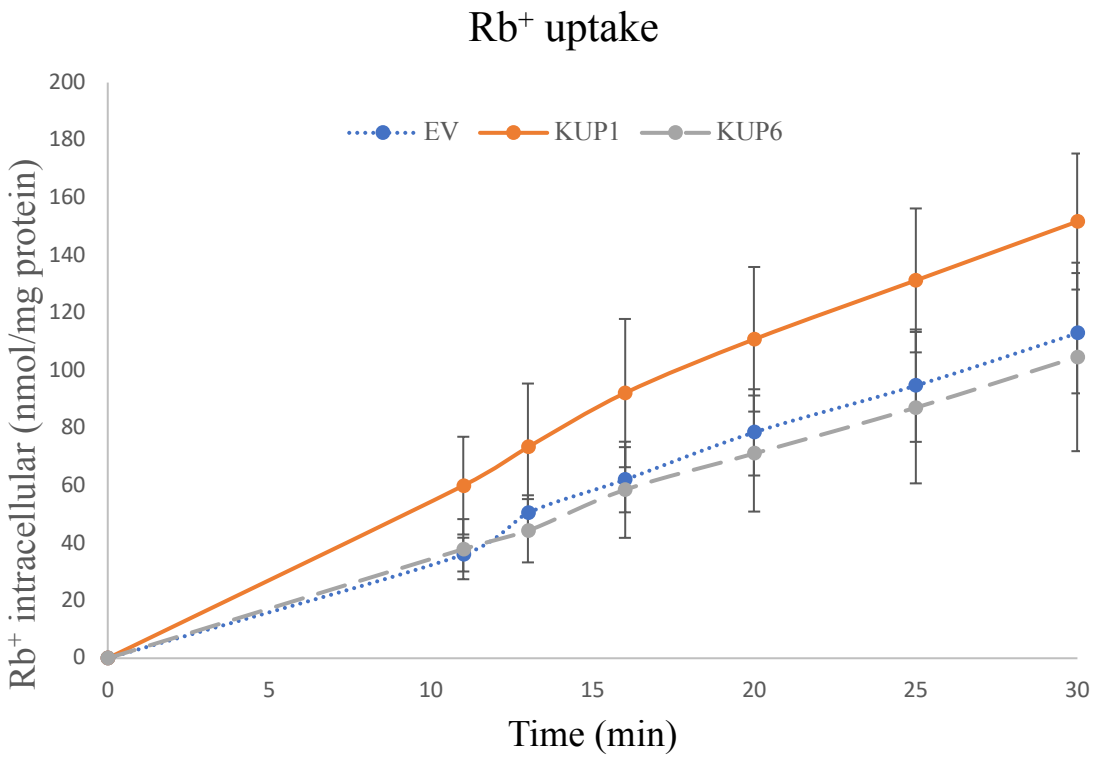


Figure 32. Rb+ uptake activity

How it can see in fig. 31 and 32, the error bars are very large. As for the complementation test, the reasons can be different as contaminations, mistakes during the test, different condition. It's impossible to replicate the experiment at the same condition each time. There are several variables that can affect the assay.

4.4 Potassium Efflux experiment

The efflux, in contrast with the uptake, shows the decrease of K^+ concentration within the cells.
The diagram below shows the efflux ability of EV, KUP1 and KUP6 using different pH.
At time zero on the diagram, the concentration of K is high and then it decreases until it becomes stable.
Every diagram shows the average value obtained after three repetitions.
As it's clearly visible, the efflux activity is not strong either for KUP1 or KUP6.
Even if KUP6 shows a light activity with pH6 and pH7,5.
Instead both of them had a well activity when the assay was performed with HEPES NaOH Ph 8,0 - 0,4 M NaCl.

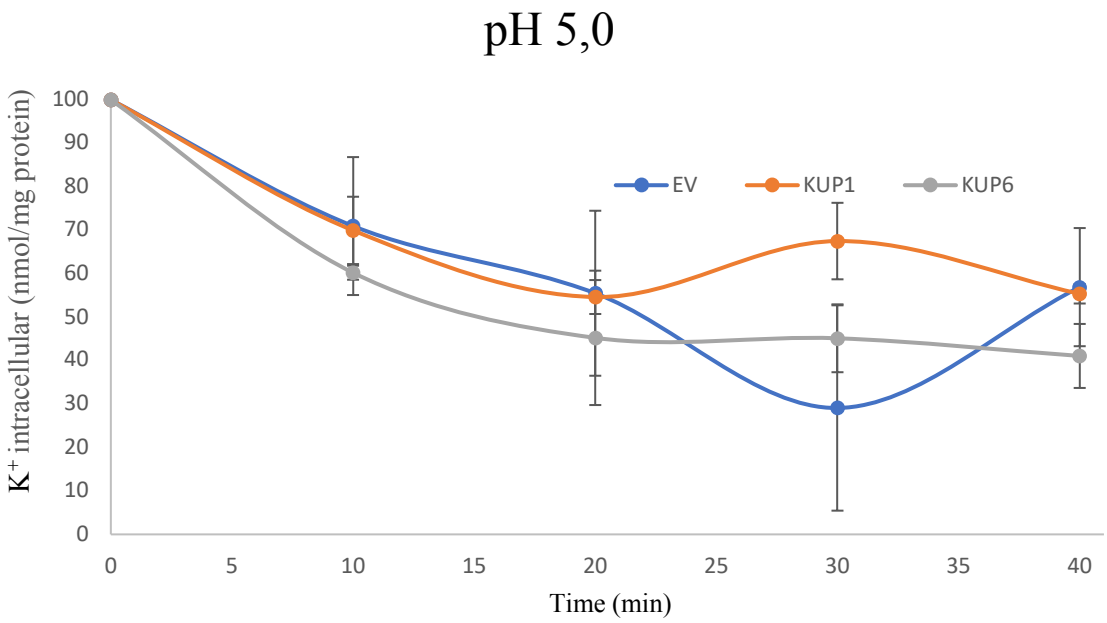


Figure 33. K+ efflux activity pH 5,0

Fig. 33 shows the result using pH 5,0. The gap between the various strains is really small. That means there isn't efflux activity.

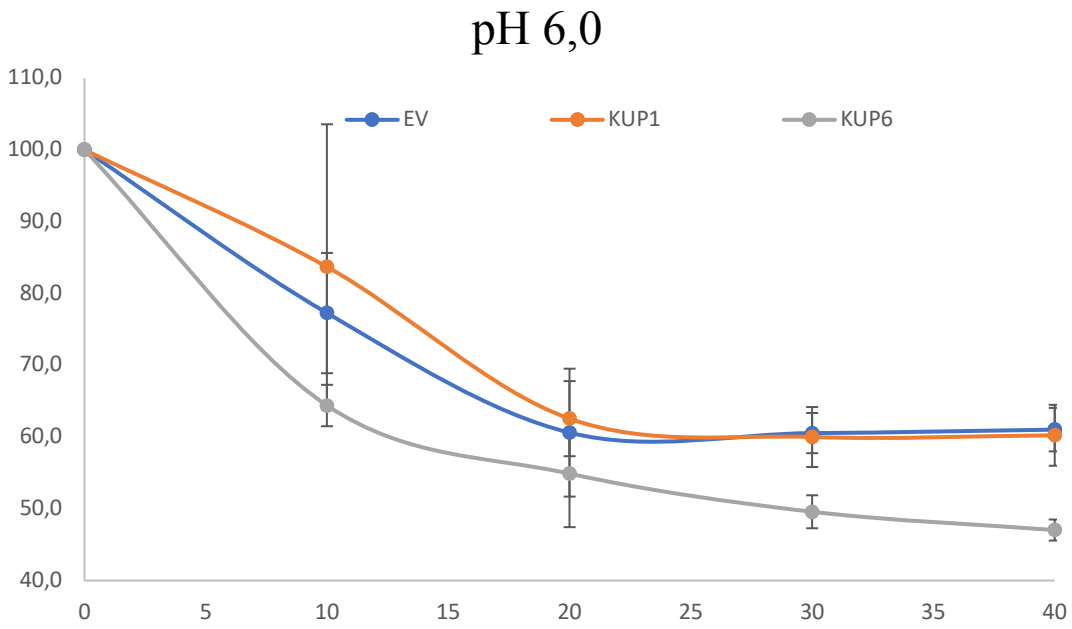


Figure 34. K+ efflux activity pH 6,0

Fig. 34 shows the results using pH 6,0. In this case *At*KUP6 shows efflux activity even if it's still low. *At*KUP1 doesn't show any efflux activity indeed it can see the curve of EV (blue) and *At*KUP1(orange) overlapped.

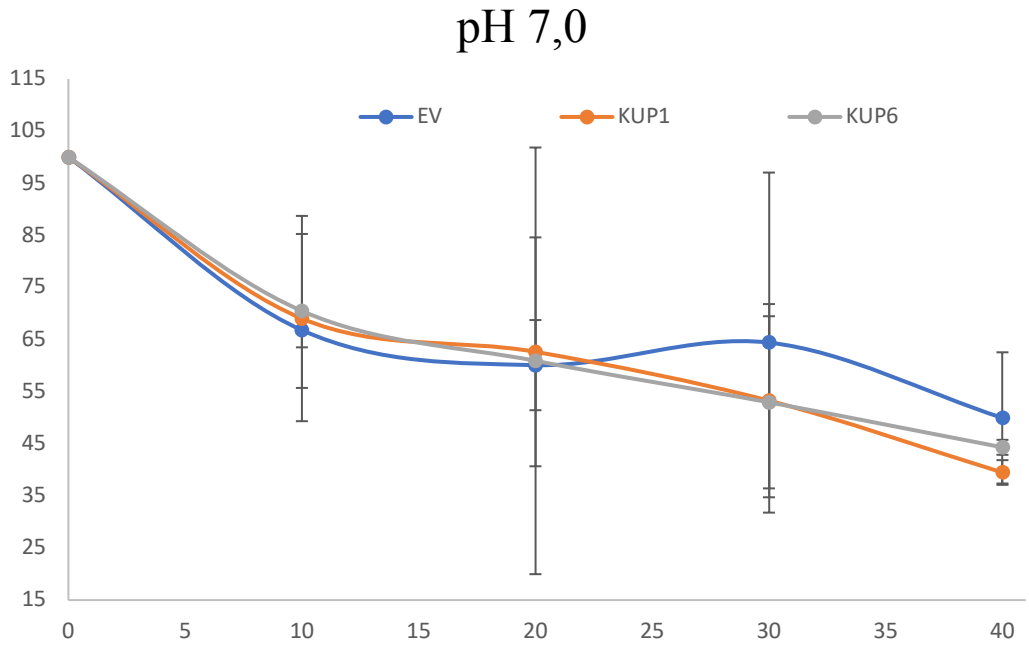


Figure 35. K+ efflux activity pH 7,0

Fig. 35 shows the results using pH 7,0. The error bars are really large so the data aren't probably accurate. It was replaced several times but each time the values didn't match with the previous test. Neither *At*KUP1 or *At*KUP6 show efflux activity.

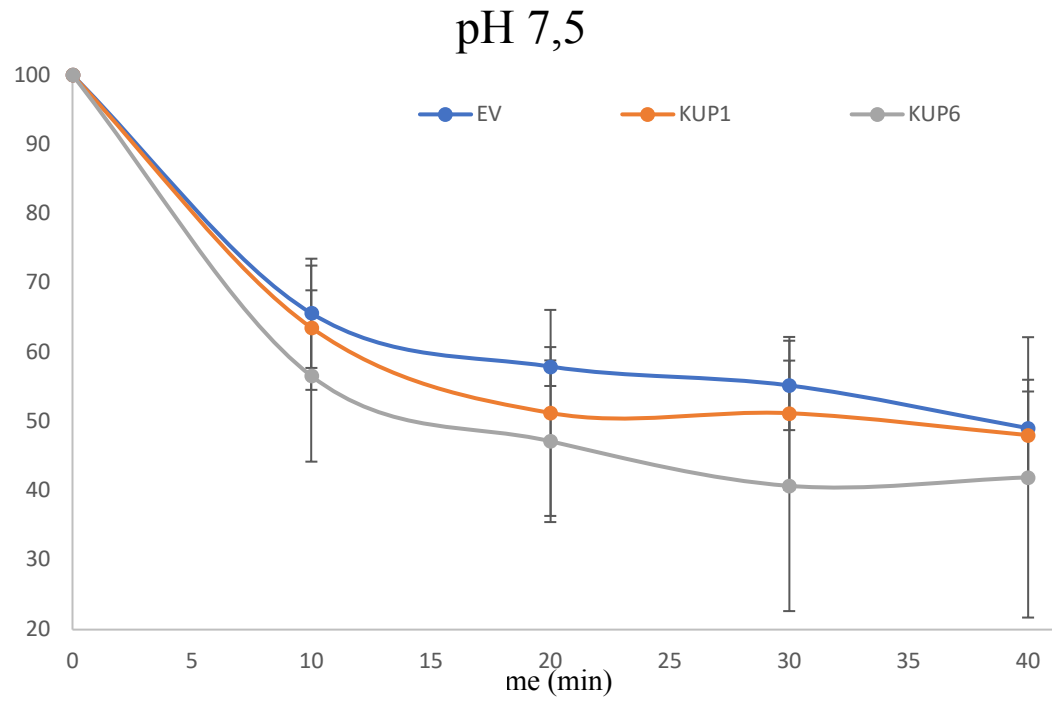


Figure 36. K+ efflux activity pH 7,5

Fig. 36 shows the results using pH 7,5. It can see the trend of curves is almost the same as pH 6,0. *At*KUP6 shows efflux activity even if it's low. *At*KUP1 doesn't show any efflux activity indeed it can see the curve of EV and *At*KUP1overlapped.

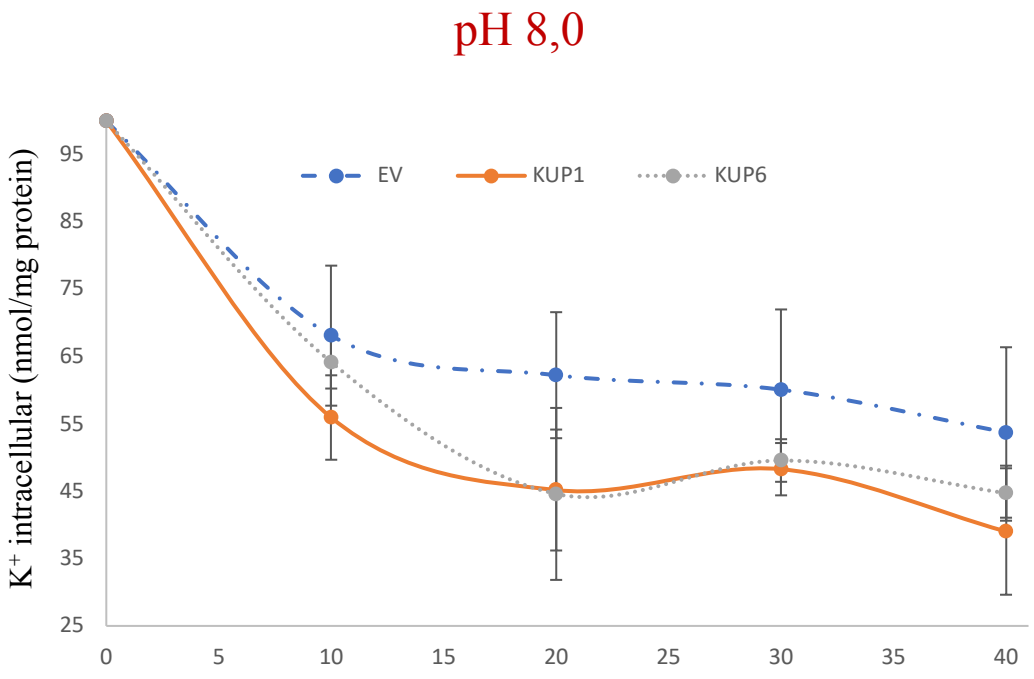


Figure 37. K+ efflux activity pH 8,0

Fig. 37 shows the results using pH 8,0. The best results have been obtained with this condition.

The gap between EV and *At*KUP1/KUP6 is higher compared to the other pH values .
The concentration of K^+ decreases exponentially and, after 15 minutes, it becomes steady.
Fig. 38 shows the trend of data in the first twenty minutes. The decrease of concentration is shown. *At*KUP1 shows a higher uptake activity than *At*KUP6. Although both of them have a good efflux activity compared to EV.

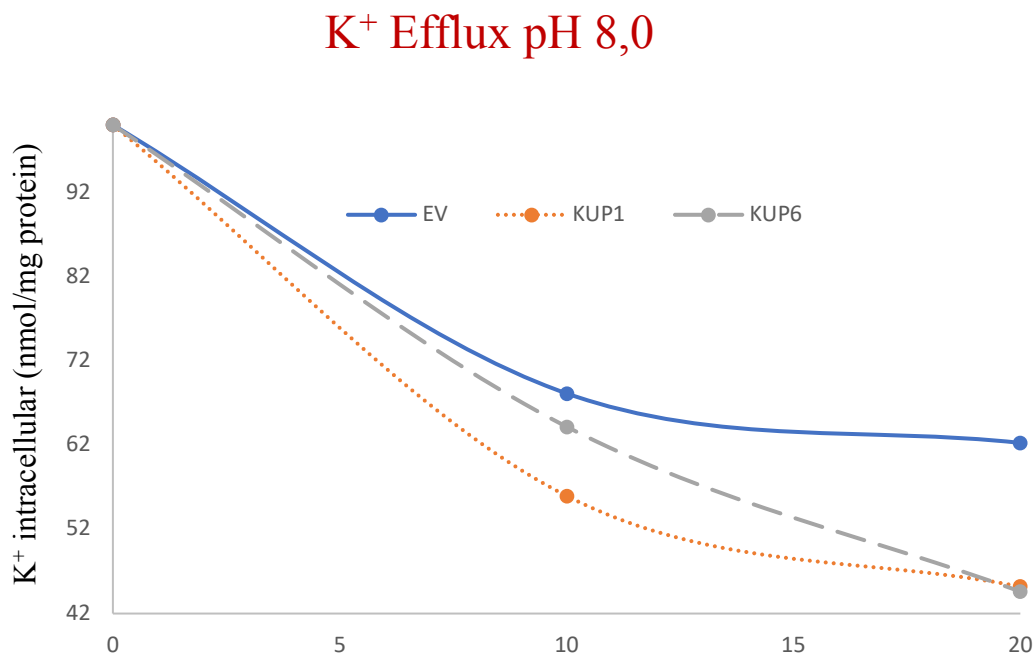


Figure 38. K+ efflux activity pH 8,0 (zoom)

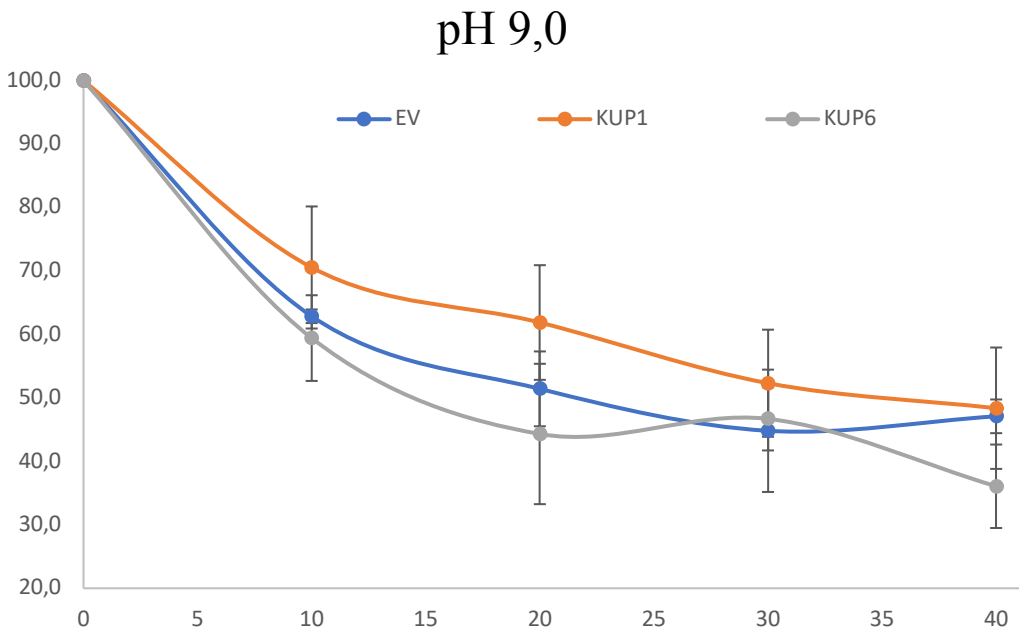


Figure 39. K+ efflux activity pH 9,0

Fig. 39 shows the results using pH 9,0. Also in this case there isn't efflux activity.
The *At*KUP1 curve is even above the EV curve. That means EV has a higher activity than *At*KUP1 even if it's strange.

5. Conclusions

In conclusion after the efflux and uptake activities were analyzed several times and by varying conditions as buffer, pH and time of incubation, it was showed that:

- *At*KUP1 has a strong uptake activity and it is also able to uptake different ions. It's not specific for only one ion. It's efflux activity compared with the uptake activity is low and it shows it only at pH 8. Furthermore, it's able to complement the growth of *E. coli* LB2003 within specific K^+ concentration [K20++(20 mM K^+), K10++ (10 mM K^+), K5+ (5mM K^+), K1 (1 mM K^+)].
- *At*KUP6 hasn't neither a strong uptake activity or efflux activity. It's not able to uptake the ions we tested (K^+, Cs^+, Li^+, Rb^+). The efflux activity can be see with three different range of pH even if it's every time low but still higher than *At*KUP1 efflux activity. As regards to the growth activity it's still able to complement the growth of *E. coli* LB2003 but its ability is lower than *At*KUP1 which means the growth is slower with this strain at the same growth condition.

6. References

Pascal Mäser, Markus Gierth & Julian I. Schroeder, 2002, Molecular mechanisms of potassium and sodium uptake in plants, *Plant and Soil* **247**, 43-54.

Ellen Tanudjaja, Naomi Hoshi, Yi-Hsin Su, Shin Hamamoto and Nobuyuki Uozumi, 2017, Kup-mediated Cs^+ uptake and Kdp-driven K^+ uptake coordinate to promote cell growth during excess Cs^+ conditions in *Escherichia coli*, Scientific Reports [DOI:10.1038/s41598-017-02164-7].

Mark W. Szczerba, Dev T. Britto, Herbert J. Kronzucker, 2009, K^+ transport in plants: Physiology and molecular biology, Journal of Plant Physiology **166**, 447-466.

David W. Meinke, J. Michael Cherry, Caroline Dean, Steven D. Rounsley, Maarten koorneef,1998, *Arabidopsis thaliana*: A Model Plant for Genome Analysis, *Science* **282**, 662-682.

Nobuyuki Uozumi, 2001, *Escherichia coli* as an expression system for K^+ transport systems from plants, *Am J Physiol Cell Physiol* **281**, C733-C739.

Yuriko Osakabe, Naoko Arinaga, Taishi Umezawa, Shogo Katsura, Keita Nagamachi, Hidenori Tanaka, Haruka Ohiraki, Kohji Yamada, So-Uk Seo, Mitsuru Abo, Etsuro Yoshimura, Kazuo Shinozaki and Kazuko Yamaguchi-Shinozaki, 2013, Osmotic Stress Responses and Plant Growth Controlled by Potassium Transporters in *Arabidopsis*, *The plant Cell* **25**, 609-624.

Alexander Grabov, 2007, Plant KT/KUP/HAK Potassium Transporters: Single Family – Multiple Functions, *Annals of Botany* **99**, 1035-1041.

Stanislav Isayenkov and Frans J.M. Maathuis, 2013, *Arabidopsis thaliana* vacuolar TPK channels form functional K^+ uptake pathways in *Escherichia coli*, *Plant Signaling & Behavior* **8**: 7, e24665.

Eugene J. Kim, June Myoung Kwak, Nobuyuki Uozumi, and Julian I. Schroeder, 1998, *At*KUP1: An *Arabidopsis* Gene Encoding High-Affinity Potassium Transport Activity, *The Plant Cell* **10**, 51-62.

David L. Nelson and Michael Cox, 2016, Principles of Biochemistry, Chap. 11. WH Freeman [ISBN-10 1319108245; ISBN-13 978-1319108243].

P. K. Smith, R. I, Krohn, G. T, Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, 1985, Measurement of protein using bicinchoninic acid, *Analytical Biochemistry,* Volume 150.

Suliman Khan, Muhammad Wajiid Ullah, Rabeea Siddique, Ghulam Nabi, Sehrish Manan, Muhammad Yousaf and Hongwei Hou, 2016, Role of Recombinant DNA Technology to Improve Life, *International Journal of Genomics*, Article ID 2405954.

T. W. Overton, 2014, Recombinant protein production in bacterial hosts, Drug Discovery Today **19**, 590-601.

R. Chen, 2012, Bacterial expression systems for recombinant protein production: *E. coli* and beyond, Biotechnology Advances **30**, 1102-1107.

Masami Inaba, Atsushi Sakamoto and Norio Murata, 2001, Functional Expression in *Escherichia coli* of Low-Affinity and High-Affinity $Na^+(Li^+)/H^+$ Antiporters of *Synechocystis*, Journal of Bacteriology, 1376-1384.

German L. Rosano and Eduardo A. Ceccarelli, 2014, Recombinant protein expression in *Escherichia coli:* advances and challenges, Frontiers in Microbiology **5**, 172.

Brian Pope and Helen M. Kent, 1996, High efficiency 5 min transformation of *Escherichia coli*, Oxford University Press, Vol. 24, 536-537.

Alonso Rodriguez-Navarro, 2000, Potassium transport in fungi and plants, Biochimica et Biophysica Acta **1469**, 1-30.

Martha V. Radchenko, Kimihiro Tanaka, Rungaroon Waditee, Sawako Oshimi, Yasutomo Matsuzaki, Masahiro Fukuhara, Hiroshi Kobayashi, Teruhiro Takabe, and Tatsunosuke Nakamura, 2006, Potassium/Proton Antiport System of *Escherichia coli*, The Journal of Biological Chemistry Advances **281**, 19822-19829.

John J. Sheahan, Leonidio Ribeiro-Neto and Michael R. Sussman, 1993, Caesium-insensitive mutants of *Arabidopsis thaliana*, The Plant Journal **3** (5), 647-656.

Roderick MacKinnon, 2004, Potassium Channels and the Atomic Basis of Selective Ion Conduction, Angew. Chem. Int. Ed. **43**, 4265-4277.

Bruce Alberts, Dennis Bray, Karen Hopkin, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, Peter Walter, 2011, L'essenziale di Biologia molecolare della cellula, Chap. 12, Zanichelli [ISBN 978-88-08-06221-5].

Marschner H., 1995, Mineral nutrition of higher plants, 2nd edition San Diego, Academic Press.

Fu HH, Luan S., 1998, *At*KUP1: a dual-affinity K^+ transporter from *Arabidopsis*, Plant Cell **10**, 63-73.

Ahn SJ, Shin R, Schachtman DP., 2004, Expression of *KT/KUP* genes in *Arabidopsis* and the role of root hairs in K^+ uptake, Plant Physiol **134,** 1135-1145.

Ashley MK, Grant M, Grabov A., 2006, Plant responses to potassium deficiencies: a role for potassium transport proteins, J Exp Bot **57**, 425-436.

Shabala S, Cuin TA, 2008, Potassium transport and plant salt tolerance, Physiol Plant **133,** 651-669.

Hampton, C. R. *et al*, 2004, Caesium Toxicity in *Arabidopsis,* Plant Physiol **136,** 3824-3837.

Uozumi Nobuyuki, Gassmann W., Cao Y. & Schroeder J. I., 1995, Identification of strong modifications in cation selectivity in an *Arabidopsis* inward rectifying potassium channel by mutant selection in yeast, J. Biological Chemistry **270,** 24276-24281.

Dirk Bossemeyer, Andreas Schlosser, and Evert P. Bakker, 1989, Caesium Transport via the *Escherichia coli* Kup (TrkD) Uptake System*,* Journal of Bacteriology **171,** 2219-2221.

Rangasamy P. Elumalai, Punita Nagpal, and Jason W. Reed, 2002, A mutation in the *Arabidopsis KT2/KUP2* Potassium Transporter Gene Affects Shoot Cell Expansion*,* The Plant Cell **14,** 119-131.

Yoko Sato, Kei Nanatami, Shin Hamamooto, Makoto Shimizu, Miho Takahashi, Mayumi Tabuchi-Kobayashi, Akifumi Mizutani, Julian I. Schroeder, Satoshi Souma and Nobuyuki Uozumi, 2014, Defining membrane spanning domains and crucial membrane-localized acidic amino acid residues for K^+ transport of a Kup/HAK/KT-type

Escherichia coli potassium transporter, J. Biochem **155**(5), 315-323.

Fernando Aleman, Fernando Caballero, Reyes Rodenas, Rosa M. Rivero, Vicente Martinez and Francisco Rubio, 2014, The F130S point mutantion in the *Arabidopsis* in high-affinity K^+ transporter AtHAK5 increases K^+ over Na^+ and Cs^+ selectivity and confers Na^+ and Cs^+ tolerance to yeast under heterologous expression, frontiers in Plant Science [DOI: 10.3389/fpls.2014.00430]

Nobuyuki Uozumi, Eugene J. Kim, Francisco Rubio, Takao Yamaguchi, Shoshi Muto, Akio Tsuboi, Evert P. Bakker, Tatsumosuke Nakamura, and Julian I. Schroeder, 2000, The *Arabidopsis* HKT1 Gene Homolog Mediates Inward Na^+ Currents in *Xenopus Iaevis* Oocytes and Na^+ Uptake in *Saccharomyces,* Plant Physiology **122,** 1249-1259.

Daisuke Kobayashi, Nobuyuki Uozumi, Shun'ichi Hisamatsu, and Mtsumi Yamagami, 2010, *At*KUP/HAK/KT9, a K^+ Transporter from *Arabidopsis thaliana,* Mediates Cs^+ Uptake in *Escherichia coli,* Biosci. Biotechnol. Biochem. **74**(1), 203-205.

Stanley G. Schultz and A. K. Solom, 1961, Cation Transport in *Escherichia coli,* The Journal of General Physiology **45,** 355-368.

R. Garcia and A. P. Baez, 2012, Atomic Absorption Spectrometry (AAS), Atomic Absorption Spectroscopy, Dr. Muhammad Akhyar Farrukh (Ed.) [ISBN: 978-953-307-817-5].

LIST OF FIGURES

Figura 1. Animal cell plasma membrane (Encyclopaedia Britannica/UIG/Getty Images) 1
Figura 2. Lipid bilayer (http://images.slideplayer.com/17/5357157/slides/slide_4.jpg) 2
Figure 3. Synthetic double lipidic layer without proteins (left) and plasma membrane (right). 2
Figura 4. . Double layer permeability based on the molecules dimensions. (Zanichelli, 2011). 4
Figura 5. Passive or Active Transport, Channel or Carrier Protein (Garland Science 2014) 5
Figura 6. Carriers classification 5
Figura 7. (ddgene.org/plasmids-101-what-is-a-plasmid) 7
Figura 8. Example of host cell (University of Wakaito, 2014) 7
Figura 9. E. coli LB2003 (mutant) 8
Figura 10. E. coli TO114 (mutant) 8
Figura 11. . Plant cell (http://www.bbc.co.uk/staticarchive/9b7156a275ad2a777763855f15f5446dfeb57c90.gif) 9
Figura 12. A. thaliana growing in the laboratory 9
Figura 13. From Molecular mechanisms of potassium and sodium uptake in plants- Pascal Mäser, Markus Gierth & Julian I. Schroeder 10
Figura 14. Curve of growth (https://www.peakprosperity.com/forum/population-growth-s-shaped-not-exponential/7250) 12
Figura 15. Important elements for plants (Petra Marschner, 1986) 13
Figura 16. Comparison of growth of the K+ uptake system-deficient E. coli strain LB2003 transformed with Kup or EV at various concentrations of K+ and Cs+ 14
Figura 17. DNA cloning (University of Waikato, 2007) 15
Figura 18. LBK300 solid and liquid medium 19
Figura 19. 2 mL KLB300+ Amp 20
Figura 20. Sample stocks at -80°C 20
Figura 21. Culture 20
Figura 22. BCA protein assay kit 35
Figure 23. 96 well plates after 1 hr of incubation 36
Figura 24. Results of first assay [K20++(20 mM K^+), K10++ (10 mM K^+), K5+ (5mM K^+), K1 (1 mM K^+), EV (empty vector), EcKUP (E. coli with all K^+ transporters), KUP1 (A. thaliana KUP1) and KUP6 (A. thaliana KUP6)] 38
Figura 25. Results of second assay [K20++(20 mM K^+), K10++ (10 mM K^+), K5+ (5mM K^+), K1 (1 mM K^+), EV (empty vector), EcKUP (E. coli with all K^+ transporters), KUP1 (A. thaliana KUP1) and KUP6 (A. thaliana KUP6)] 39
Figura 26. Results of first assay [K20++(20 mM K^+), K10++ (10 mM K^+), K5+ (5mM K^+), K1 (1 mM K^+), EV (empty vector), EcKUP (E. coli with all K^+ transporters), KUP1 (A. thaliana KUP1) and KUP6 (A. thaliana KUP6)] 40
Figure 27. Growth test performed by adding k^+ 41
Figure 28. Growth test by combining K^+ and Cs^+ 42
Figure 29. K^+ uptake activity 43

Figure 30. Cs+ uptake activity 44
Figure 31. Li+ uptake activity 45
Figure 32. Rb+ uptake activity 45
Figure 33. K+ efflux activity pH 5,0 46
Figure 34. K+ efflux activity pH 6,0 47
Figure 35. K+ efflux activity pH 7,0 47
Figure 36. K+ efflux activity pH 7,5 48
Figure 37. K+ efflux activity pH 8,0 48
Figure 38. K+ efflux activity pH 8,0 (zoom) 49
Figure 39. K+ efflux activity pH 9,0 49

LIST OF TABLES

Table 1. 10% KO stock solution 24
Table 2. KCl rich medium composition 24
Table 3. Solutions added to the medium 25
Table 4. Example of absorbance values after 7 hours (d10x: 10uL of water + 90uL of cells suspensions) 25
Table 5. example of value of 100 times dilution 26
Table 6. Optical density values 27
Table 7. Medium composition 30
Table 8. Example of values 31
Table 9. Buffers that have been used 32
Table 10. Samples composition 34
Table 11. Eluition Buffer composition 36
Table 12. Standards composition 36
Table 13. Optical density measured at 600 nm (OD600) 41
Table 14. Optical density measured at 600 nm (OD600) 42

LIST OF SYMBOLS

At = *Arabidopsis Thaliana*
Ec = *Escherichia coli*
K^+ = Potassium
Cs^+ =Caesium
Rb^+ = Rubidium
Li^+ = Litium
*Ec*Kup = *Escherichia coli* K^+ uptake
KUP/HAK/KT = high-affinity K^+ symporter family
HKT = high-affinity K^+ transporter
MES = 2-(*N*-morpholino)ethanesulfonic acid

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
TRIS = 2-Amino-2-(hydroxymethyl)propane-1,3-diol
CHES = 2-(Cyclohexylamino)ethanesulfonic acid
LB2003 = F^- *kup1 ΔkdpABC5 ΔtrkA rpsL metE thi rha gal*
TO114 = W3110 *nhaA*::Km^r *nhaB*::Em^r *chaA*::Cm^r
EV = empty vector
K1, K5, K10 & K20 = Potassium concentration of 1, 5, 10 & 20 mM
Amp = Ampicillium
IPTG = Isopropyl β-D-1-thiogalactopyranoside
MQ water = Milli-Q water (purified water)
LBK = potassium-modified Luria broth
EDTA = Ethylenediamenetetraacetic acid
TCA = Trichloroacetic acid
AAS = Atomic Absopition Spectrometry
OD = optical density
BCA = biocinchoninic acid

Acknowledgements

I would like to thank my supervisor, Uozumi sensei, for his kindness, support and help during my permanence in his laboratory.

I would like to thank also my supervisor in Italy, prof. Francesca Bosco, for her valuable guidance throughout this thesis.

Further, I would render thanks to Ellen Tanudjaja and Masaru Tsuji for the gentle assistance during long hours in laboratory and for their temper. Their help was essential to provide and analyze many data and to perform my experiments.

I thank also my lab mates at the department of biomolecular engineering for taking me in.

I'm very thankful for Politecnico di Torino and Tohoku University that gave me the chance to do my master thesis abroad.

If today I have achieved my aim it's thank to the support of many important people in my life.

My colleagues Chiara, Melania, Elga, Fabio and Enrico that are more than classmates, they are friends and thanks to them the long days at the university weren't so hard.

My lifelong friends Valeria, Federica, Giada and Giuseppe. Even if they were far away they were important and supportive during my studies.

Giacomo to always be by my side and put up with me during tough moments and years.

Monica San, my favorite adventure fellow. She was a perfect mate event thought she is always late. Without her my experience in Japan wouldn't be the same. I found a beautiful friend.

Mirza for his kindness and to always make me smile. He helped me very much with my English and in his own little, he supported me during my experiments.

Thank to Marco, Filippo, Robin, Anton, Rickard, Manon and Malin. They were like a family, my Japanese family, mates of thousand adventures and misadventures.

Last but not least I have to thank my family. My sister, Sofia, to be my fan number one and my parents to believe in me even when I don't do it. They are my mentors and my cornerstones. Without them I couldn't have done anything. Their support and faith were the most important thing in these tough years. They gave me everything and I hope they can be proud of me today.